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PRINCIPAL INVESTIGATOR: Gail E. Sonenshein, Ph.D.

CONTRACTING ORGANIZATION: Boston University
Boston, Massachusetts 02118

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Recent evidence suggests that the rise in breast cancer rates reflects increased exposure to and bioaccumulation of environmental pollutants, such as 7,12-dimethylbenz(*a*)anthracene (DMBA). Female Sprague-Dawley rats treated with DMBA develop mammary (breast) tumors by about 15 weeks. Here we proposed to elucidate the roles of the aromatic hydrocarbon (Ah) and estrogen receptors and the c-*myc* oncogene. The hypothesis investigated was that administration of DMBA would lead to AhR-mediated induction of P-450 enzymes. The resulting oxidative stress is known to activate NF-κB/Rel transcription factors, leading to enhanced c-*myc* expression and cell survival. Many aspects of this model have been confirmed: 1. Aberrant activation of NF-κB typified rat and and human breast cancers; 2. c-*myc* RNA levels were elevated in mammary tumors; 3. AhR mRNA and protein levels were elevated in rat and human breast cancer; 4. Inhibition of AhR slowed growth of breast cancer cells; 5. Inhibition of NF-κB/Rel resulted in apoptosis of breast cancer cells; 6. Activation of NF-κB in mammary glands following DMBA administration preceded tumor formation; 7. A novel transcription factor complex containing AhR and the p65 subunit of NF-κB activated transcription of the c-*myc* promoter. These findings suggest AhR and NF-κB function to promote proliferation, survival and neoplastic transformation of breast cancer, and represent potential novel targets for therapeutic intervention in breast disease.

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Carl L. Bonewald 7/25/99

PI - Signature Date

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5) INTRODUCTION

Recent evidence suggests that the rise in breast cancer rates reflect increased exposure to and bioaccumulation of environmental pollutants, including polycyclic aromatic hydrocarbons such as 7,12-dimethylbenz(*a*)anthracene (DMBA). DMBA treatment of female Sprague-Dawley rats results in induction of mammary gland (breast) tumors. Mammary gland tumors, which develop by about 15 weeks after a single dose of DMBA, are generally well-differentiated and retain their hormonal responsiveness. In this application, we proposed to elucidate the roles of the receptors for the aromatic hydrocarbons, estrogen and prolactin hormones and the c-*myc* oncogene in a systematic, integrated fashion. Studies were proposed to evaluate changes in the expression and function of the aromatic hydrocarbon (Ah) and hormone receptors in normal and malignant tissue, and the regulation of any altered phenotype. We also proposed to monitor for potential dys-regulated expression of the c-*myc* oncogene, and elucidate the mechanisms mediating these changes. Multiple lines of evidence, including studies in transgenic mice, have demonstrated that the c-*myc* oncogene plays an important role in regulating changes that lead to neoplastic transformation of mammary epithelial cells. Progress has been made on all of the specific aims. Of note, our evidence support a role for the aberrant activation of the NF-κB/Rel family of transcription factors by aromatic hydrocarbon receptor signaling in the tumors, and suggest a novel mechanism whereby these transcription factors play a role in dysregulation of the c-*myc* gene. These findings have important implications with respect to potential new therapeutic approaches as well as the development of new biologic markers for characterization of pre-malignant tissue. In this report, an introduction is given first to set the stage with regards to the rat model, as well of the role of the Ah and estrogen receptors and of the expression of the c-*myc* oncogene in neoplastic transformation. Lastly, a brief introduction to the NF-κB/Rel family is also given.

Mammary Gland Tumorigenesis in Rats

It has been estimated that known risk factors such as genetics, diet, and endogenous estrogen levels account for approximately 30% of human breast cancer cases (1,2). However, changes in these factors appear unable to explain the apparent recent increases in breast cancer mortality and incidence. It has been suggested that some of the rise in breast cancer rates reflects increased exposure to and bioaccumulation of lipophilic environmental pollutants such as polycyclic aromatic hydrocarbons (PAH) like DMBA and related organochlorines (1,2). This conclusion has been drawn in part from epidemiologic studies associating elevated breast cancer rates with PAH exposure (3-6) and from studies demonstrating increased levels of aromatic hydrocarbons in breast carcinomas (7,8), and in sera from breast cancer patients (5). Given the potentially critical role that PAH may play in human breast cancer, the use of the DMBA/rat mammary tumor model to study tumorigenesis should be viewed as particularly relevant to human disease.

Rat models for breast cancer are widely used. The DMBA single dose model has permitted elucidation of factors that act at initiation of neoplasia in the gland and at the subsequent steps on tumor development. Review of papers from our own and other laboratories shows good agreement on the characteristics of tumorigenesis induced in female Sprague-Dawley (S-D) rats by DMBA with only the variations that can be expected on the basis of biological variability or differences in protocols (9-21). Under the auspices of the International Life Sciences Institute (ILSI), Dr. Rogers and 4 other experts on the DMBA model made a comprehensive review and evaluation of the model and of tumors induced (16). The mammary

gland tumors induced in rats comprise a spectrum of morphology from benign, typical fibroadenomas and adenomas to papillomas with hyperplastic, atypical or dysplastic epithelium and significant stromal and myoepithelial components, to tumors that are architecturally and cytologically malignant and invade adjacent normal tissue. Metastases from even the most anaplastic tumors are rare (16).

Aromatic Hydrocarbon Receptor-Dependent Tumorigenesis

The most proximal event in PAH tumorigenesis is the binding of environmental chemicals such as DMBA, benzo[*a*]pyrene, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and polychlorinated biphenyls (PCBs) to a cytosolic aromatic hydrocarbon receptor (AhR) (22-24). Therefore, rather than scrutinize the toxicology of a myriad of carcinogenic chemicals (e.g. there are over 200 possible PCB isomers and congeners) it is prudent to focus on a common, critical element in the transformation pathway, AhR activation. Molecular analysis of AhR structure and function has only recently been facilitated by the cloning of the murine AhR gene (22,23) and the gene coding for a requisite accessory molecule called "Arnt" (25). The AhR and Arnt are highly conserved throughout evolution (24-26).

Like most other members of a family of DNA-binding protein receptors (e.g. the estrogen, progesterone, and glucocorticoid receptors), unbound AhR is associated with a heat shock protein which may repress receptor nuclear translocation (27). Upon binding with PAH, hsp90 dissociates and the receptor complexes with an accessory molecule "Arnt," the "aromatic hydrocarbon nuclear translocating" factor (24). Formation of this heterodimer appears to be mediated by helix-loop-helix motifs present on the AhR and on Arnt (22,24). Arnt-dependent AhR activation results in receptor binding to specific Ah response elements located within an enhancer element (28). AhR binding to these core recognition sequences induces transcription of a battery of genes which code for enzymes involved in PAH metabolism (29,30). A hallmark of this transcriptional activity is the induction of cytochrome P-450IA1 mRNA synthesis and P-450 enzyme activity (29-33). Oxidation of PAH by P-450 and subsequent metabolism by P-450-dependent "phase II" enzymes (e.g. UDP glucuronyltransferase, NAD[P]H:menadione oxidoreductase, aldehyde dehydrogenase, and glutathione transferase) result in the production of reactive oxygen intermediates (e.g. diol-epoxides) which form DNA adducts, induce DNA damage, and ultimately elicit cell transformation. The role of pro-oxidant production and DNA-adduct formation in human and rat mammary neoplasias following DMBA exposure has been well documented (1,11,34,35). The extent to which this pathway is activated, as measured by P-450IA1 levels, has been correlated with human cancer risk (36-39).

AhR activity may be particularly relevant to breast cancer given the documented "cross-talk" between AhR- and ER-dependent cell activation pathways. AhR ligands such as TCDD, PCBs, dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) regulate ER (40-47) or prolactin receptor (48) levels. These regulatory activities are AhR-dependent and are frequently manifest in human breast cancer cell lines. Interestingly, trichlorodibenzofuran, an AhR ligand, induces a time- and dose-dependent increase in cytosolic and nuclear ER and progesterone receptor levels despite its failure to directly bind either the ER or the PR (49). Thus, the potential role of aromatic hydrocarbons as "xenoestrogens" may in part reflect indirect activation of the ER through the AhR. Communication in the opposite direction, from the ER-dependent to the AhR-dependent pathway, has also been documented (41,50-52). That is, activation of the ER pathway increases activity of the AhR pathway.

AhR ligands also appear to promote transformation by activating intracellular factors and proto-oncogenes. For example, PAH exposure *in vitro* or *in vivo* results in amplification of the c-*erb*-2, c-*myc*, *c-fos*, *c-jun* and *Ha-ras* proto-oncogenes (92-95). The extent to which and the

mechanisms by which the AhR mediates these activities are unclear. Collectively the data suggest that binding of the AhR by ligands induces a network of interactions involving the AhR, hormone receptors, and oncogenes, such as c-*myc*. Therefore, we took an integrated approach toward analyzing regulation of these biomarkers during breast cancer development in the rat model. As described in this report, our group has found that aberrant NF-κB activation follows *in vitro* Ah adminstration. Importantly, we have also demonstrated NF-κB activation in PAH-induced mammary tumors. Lastly, we have identified a novel interaction between AhR and the p65 subunit of NF-κB that leads to the transcriptional induction of the c-*myc* gene.

Role of Estrogen Receptors in Mammary Gland Cancer

It is well established that normal growth and differentiation of the mammary gland is dependent on estrogens and prolactin (15,16,53,54). Estrogens have been implicated in the etiology and development of human breast cancer (55-57). Growth of human breast cancer is often estrogen-responsive, and the presence of estrogen receptor (ER) in human breast tumors forms the basis for the use of antiestrogens for breast cancer prevention and treatment (58). Currently, ER is used as a prognostic factor in management of breast cancer patients. However, recent evidence have not supported a role for prolactin receptor in mammary tumor formation. Thus, the proposed studies on this receptor have not been actively pursued.

The development of the hormone responsive, DMBA-induced, mammary tumor model has contributed significantly to our understanding of the role of estrogens in breast cancer (59). While administration of estrogens to ovariectomized animals stimulates tumor growth and development, ovariectomy or treatment with antiestrogens causes rapid regression of established tumors and inhibits development of new tumors in response to DMBA (56,57). It is important to note that ovarian and other hormones of pregnancy have a protective effect against DMBA-induced carcinogenesis. This protective effect is attributed to hormone-induced differentiation, which eliminates the target cell for DMBA or decreases the cellular susceptibility to tumorigenesis (15).

Approximately one third of all human breast cancer patients respond to some form of hormone therapy. DMBA-induced tumors express ER, and progesterone receptors (PR) (57,58,60,61). The hormone responsiveness of DMBA-induced tumors provides a model for investigation of the role of hormones contributing to tumorigenesis.

Estrogens regulate cell growth and function by modulating gene expression through binding of the activated hormone-receptor complex to its responsive elements (ERE). Functional studies have demonstrated that ER consists of several functional domains (62,63). The N-terminal region (region A/B) contains a transactivation domain and is thought to be important for gene and cell specificity (62,63). The DNA binding domain is essential for ER recognition and specific binding to the ERE. The DNA-binding domain is also involved in nuclear translocation, dimerization and transactivation (62). Deletion of the DNA binding region or mutations in the dimerization domain or the transactivation domains eliminate ER function (63). Mutations in the hormone binding domain reduce ER function or result in a constitutively active receptor even in the absence of hormone. These mutations have been described in several human breast cancers (64). It is unknown whether exposure of mammary gland cells to PAH produce structural or functional changes in the estrogen and progesterone receptors.

The c-Myc Oncogene

The c-*myc* oncogene, the cellular homologue of the transforming gene of the avian myelocytomatosis virus, has been implicated in control of cell proliferation, neoplastic

transformation, and more recently in apoptosis or programmed cell death. The c-*myc* gene encodes two phosphoproteins, initiated at independent translational start sites, that localize to the nucleus (67,68). These normal cellular proteins appear to function either as activators or as repressors of transcription. They contain a region rich in basic amino acids (basic region), a helix-loop helix (HLH) and a leucine zipper (LZ) domain at the carboxy terminus (69). The Myc DNA binding consensus is as follows: CACGTG (70,71). A Myc binding partner, called Max, interacts with Myc (72); the heterodimer binds and transactivates this DNA sequence more efficiently than homodimers of Myc (70,73). In addition, c-Myc was shown to repress transcription from genes containing initiator (Inr) elements within their promoters (97,98). Inr elements were originally defined from a loose consensus 5'-YYCAYYYYY-3' found at the transcription initiation sites of genes containing upstream TATA elements (99,100).

Evidence from many laboratories, including our own, has demonstrated that c-*myc* plays a key role in control of cell proliferation, as well as neoplastic transformation (rev. in 74). In one of the first examinations of the cell cycle expression of c-*myc,* we demonstrated that c-*myc* mRNA levels are low in normal cells in quiescence and increase early during the G0 to G1 transition (75). This induction of c-*myc* expression is required for cells to enter S phase. Cycling cells maintain constant levels of c-*myc* expression (76).

Altered expression of c-*myc* is a hallmark of transformed cells (rev. in 77). Several types of genetic alterations of c-*myc* have been noted within tumors, including point mutations within exons 1 and 2, gene amplification, gene rearrangements, and retroviral insertions (68,74,77,78). As we first demonstrated several years ago, fibroblasts transformed by DMBA display overexpression of c-*myc* RNA, even when quiescent (75). This misregulation of c-*myc* expression occurred in the absence of gene rearrangement or amplification (75). Work from many laboratories, including our own (79-81), has elucidated two major sites of regulation of c-*myc* RNA expression, gene transcription and mRNA stability. Changes in the rate of c-*myc* gene transcription are responsible for many of the increases and decreases in mRNA expression (79,80).

The role of aberrant c-*myc* expression in neoplastic transformation was confirmed when transgenic mice bearing the normal and variant forms of the c-*myc* gene under the control of an immunoglobulin enhancer were found to develop B cell tumors (82). Since the tumors were of monoclonal origin, the now generally accepted model was proposed that overexpression of c-*myc* is an early event and that subsequent activation of additional oncogenes is involved in tumor formation. Mammary tumors are similarly characterized by overexpression of c-*myc*. As high as 80% of the human breast cancers in some studies were found to overexpress c-*myc* at the RNA or protein level (83-86). For many of these tumors, c-*myc* genes were present in large copy numbers indicating amplification. In others, c-*myc* genes were detected as single copies (84), indicating that the overexpression of c-*myc* was due to altered regulation. A direct role of c-*myc* in transformation of mammary cells has been demonstrated. Transgenic mice carrying c-*myc* genes under the control of promoters expressed in mammary cells, either the mammary tumor virus (MTV) (87) or the whey acidic acid (WAP) promoter (88), develop mammary tumors exclusively.

NF-κB/Rel Family of Transcription Factors

NF-κB/Rel is a family of dimeric transcription factors distinguished by the presence of a Rel homology domain (RHD) of about 300 amino acids in length which determines much of its function. Classical NF-κB is a heterodimer composed of p65 and p50 subunits (101). Other

members of the mammalian Rel family include c-Rel, p52 (also called lyt10), and RelB. The p65 and RelB, and c-Rel subunits have either potent or moderate transactivation potential, respectively, whereas, the p50 and p52 subunits bind avidly, but have only modest transactivation abilities (101). In most cells, other than B lymphocytes, NF-κB/Rel proteins are sequestered in the cytoplasm bound to one of the specific inhibitory proteins termed IκB's of which IκB-α is the paradigm. A variety of agents can induce NF-κB/Rel, including oxidative stress (102). Activation of the NF-κB/IκB complex involves phosphorylation and degradation of IκB, which allows for translocation of active NF-κB complex into the nucleus where it can bind to κB responsive elements (102). The biochemical characterization of classical NF-κB determined that it is a heterodimer. For the most part, Rel-related factors bind as hetero- or homodimers that have different activities depending on subunit composition. Genes that have been found to contain NF-κB elements are involved in control of immune function, inflammatory responses, adhesion, growth and cell survival (101). For example, our laboratory demonstrated the c-*myc* gene contains two NF-κB elements important for its expression (90,91).

6) BODY

The finding/or progress relevant to each Specific Aim is detailed in the section after the aim.

SPECIFIC AIM 1

Using the rodent model of DMBA-induced mammary tumorigenesis to investigate the pathogenesis of breast cancer: We proposed to determine the effects of DMBA on the temporal relationships between expression of the aromatic hydrocarbon and estrogen receptors, and the c-*myc* oncogene at the RNA and protein levels. For the elevated expression of c-*myc* gene, since our findings indicated a major role for NF-κB in the functional overexpression of this gene in mammary tumors, we have instead measured the level of this transcription factor using electrophoretic mobility shift analysis (EMSA). For AhR, in addition to receptor RNA and protein levels, we have monitored RNA levels for the P-450 enzyme targets of AhR gene transcription. Similarly for the estrogen receptor (ER), we have also monitored expression of its target gene, the progesterone receptor (PR).

1. Introduction

The effects of DMBA administration on gene expression in the normal and neoplastic mammary gland of female S-D rats were studied in tissues from 6 hours to 17 weeks after administration of DMBA. Part of our findings relate to the effects of tea and high fat diet; this portion was supported by a grant from the Tea Trade Health Research Association. First we will describe the results with ER and PR, and then the AhR and finally NF-κB and c-*myc*. Overall three DMBA-tumorigenesis studies were performed. In Experiment #1, rats were given deionized water to drink and the purified AIN-76A diet to eat. Body weights were recorded twice a week from start to finish of the experiment. At eight weeks of age, a single dose of 25mg DMBA/kg body weight was administered by gastric gavage. Four weeks later, biweekly palpation for tumors began. Site and size of palpable nodules were recorded as observed. Experiment #2 was identical to Experiment #1 except that 15mg DMBA/kg body weight was used. In Experiment #3, we again used 15mg DMBA/kg body weight, fed the rats deionized water and the purified

AIN-76A diet. Four days after DMBA administration, half of the rats were changed to a high N-6 polyunsaturated fatty acid (N-6-PUFA) diet containing 24% corn oil but isocalorically balanced to the purified AIN-76A diet; the other half remained on the purified AIN-76A diet (control diet). In the tumorigenesis experiments, rats were killed 16-17 weeks after DMBA or, in a few cases, earlier because they bore tumors $\geq$3 cm in size or were ulcerated. Experiment #4 was run in parallel to Experiment #3, except that rats were killed 6 hours, 24 hours, 1 week, 3 weeks or 9 weeks after DMBA exposure to assess the time course of the observed alterations.

In Experiments #1 and #2, statistical analyses of body weight gain over the observation period determined that DMBA did not alter the body weight gain of the female Sprague-Dawley rats. The expected increase in body weight gain was observed with the high N-6 PUFA diet in Experiments #3 and 4. Cumulative probability of bearing a palpable tumor was increased by the high N-6 PUFA diet independently of body weight gain in Experiment #3. These data were reported in part in the publication: Rogers, A.E., L.J. Hafer, Y.S. Iskander and S. Yang, Black tea and mammary gland carcinogenesis by 7,12-dimethylbenz(*a*)anthracene in rats fed control or high fat diets. Carcinogenesis 19: 1269 (1998).

Statistical analyses were carried out to determine if was there a relationship between body weight gain and any of the tumor endpoints (tumor number, weight, burden and latency) in Experiments #1-3. It was apparent that body weight gain did have an effect on tumor endpoints, and that endpoints should have been adjusted in Experiments #1-3; however, the original conclusion that rats fed a high N-6-PUFA diet had significantly increased tumor number, burden and weight and decreased latency compared to rats fed control diet was not altered when endpoints were adjusted accordingly. These data are reported, in part, in the following manuscript:

Rogers AE, LM Sullivan and LJ Hafer. Dietary fat, body weight and cancer: Contributions of studies in rodents to understanding these cancer risk factors in humans. manuscript submitted to Toxicological Sciences.

2. ER and PR expression in mammary tumors.

Two methodologies, receptor-ligand binding assay and immunohistochemistry, were utilized to examine the levels of ER and PR in normal mammary glands, mammary gland tumors and uteri from the experiments outlined above. Immunohistochemistry was used to examine the levels of proliferating cell nuclear antigen (PCNA) in both normal mammary glands and mammary gland tumors. PCNA is a nuclear protein that is present in cells during every stage of the cell cycle except G_0, thus it is used as an index of proliferation. Statistical analysis for significant differences in ER and PR levels between groups in all the experiments and also with age in Experiments #3 and 4 were performed.

In all three experiments, ER expression in grossly normal mammary glands of DMBA-treated rats compared to untreated controls was not significantly altered by DMBA (Fig. 1A, 1B and 1C). In Experiment #1, in which rats were given 25 mg DMBA/kg body weight, there was a suggestive increase in PR expression in the grossly normal mammary glands of DMBA-treated rats compared to controls ($p=0.07$) (Fig. 2A). However, in Experiment #2, the 15 mg/kg dose of DMBA was associated with a decreased PR expression in grossly normal mammary glands of DMBA-treated rats ($p=0.008$) compared to untreated controls (Fig. 2B). In Experiment #3, the 15 mg/kg dose of DMBA again was associated with a reduction in PR (referred to as PgR in this figure) expression in grossly normal glands, although it was not statistically significant (Fig. 2C). The high N-6-PUFA diet fed after DMBA exposure did not alter PR significantly compared to control AIN-76A diet (Fig. 2C).

DMBA-induced mammary gland tumors showed significant increases in ER (Fig. 1A and

Figure 1A.

Ex#1 Mean ER Expression in Normal and Neoplastic Mammary Glands

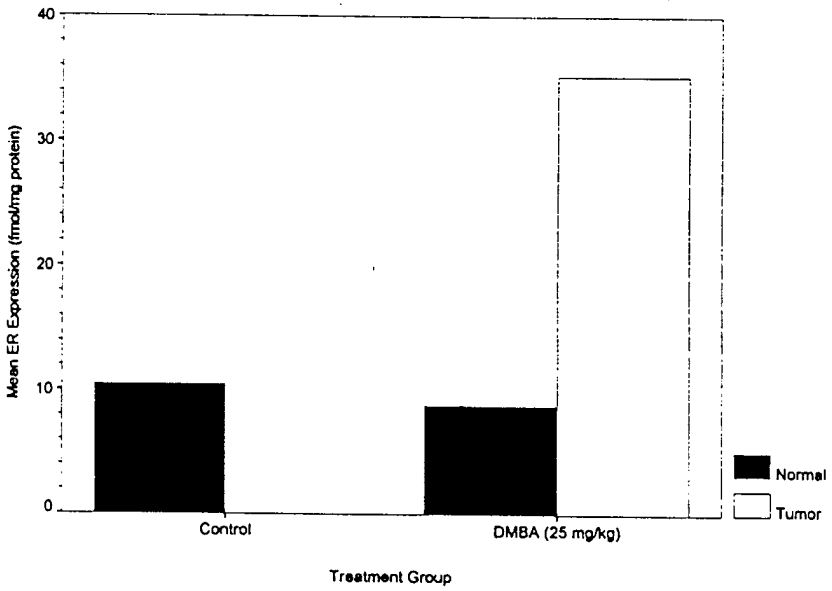

Figure 1B.

Ex#2 Mean ER Expression in Normal and Neoplastic Mammary Glands

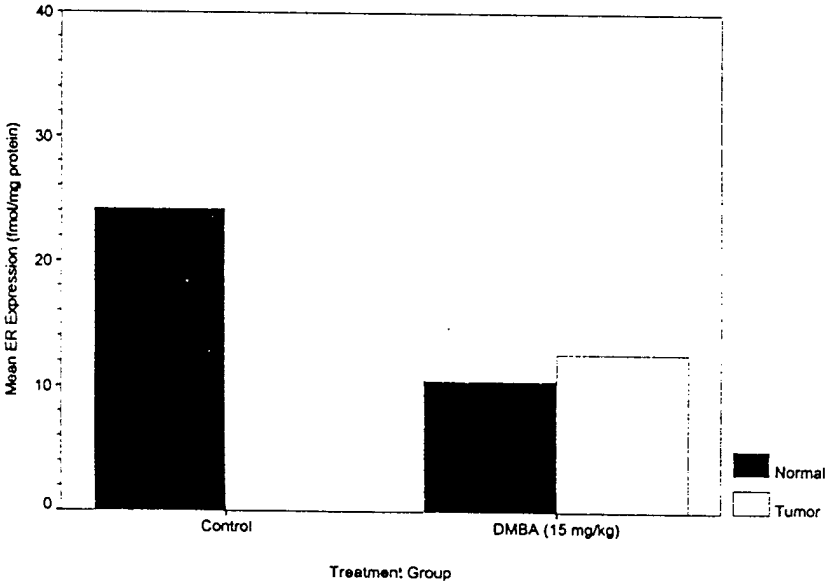

Figure 1C.

Ex#3 Mean ER Expression in Normal and Neoplastic Mammary Glands

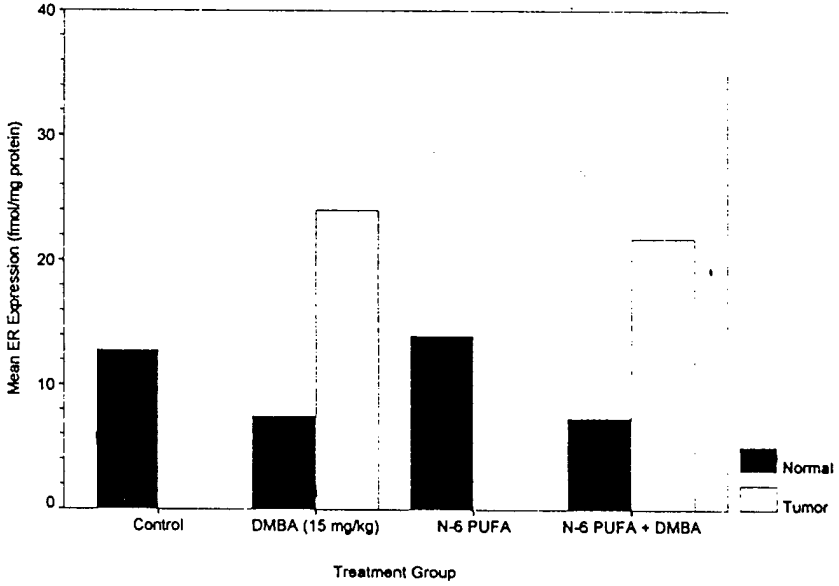

Figure 1. These charts represent the mean ER levels in grossly normal mammary glands and DMBA-induced mammary gland tumors from *(A)* Experiment #1, *(B)* Experiment #2 and *(C)* Experiment #3. There were significant increases in ER of DMBA-induced tumors versus grossly normal mammary glands in Experiments #1 and 3 ($p<0.001$); however, no significant difference was seen between tumors and normal mammary glands in Experiment #2. Control = AIN-76A diet; DMBA = AIN-76A diet + DMBA; N-6 PUFA = high N-6-PUFA diet; N-6 PUFA + DMBA = high N-6 PUFA diet + DMBA

Figure 2A.

Ex#1 Mean PgR Expression in Normal and Neoplastic Mammary Glands *

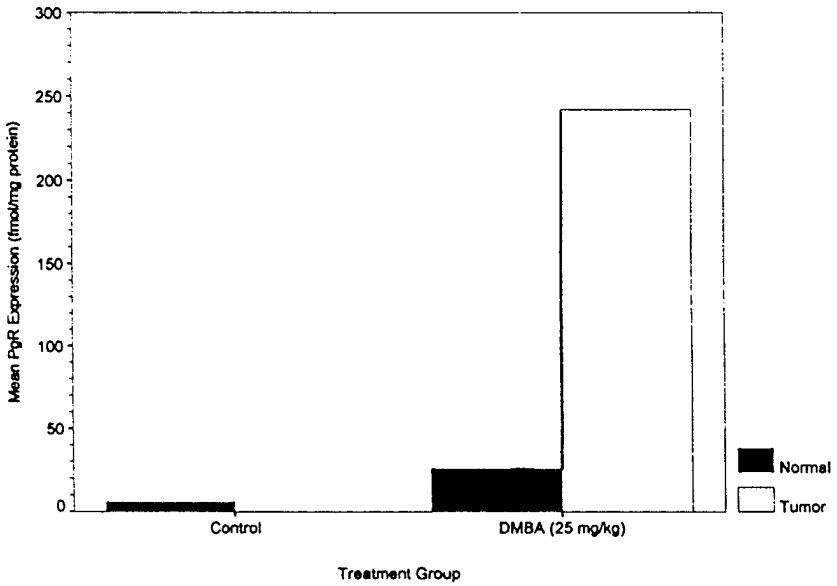

Figure 2B.

Ex#2 Mean PgR Expression in Normal and Neoplastic Mammary Glands

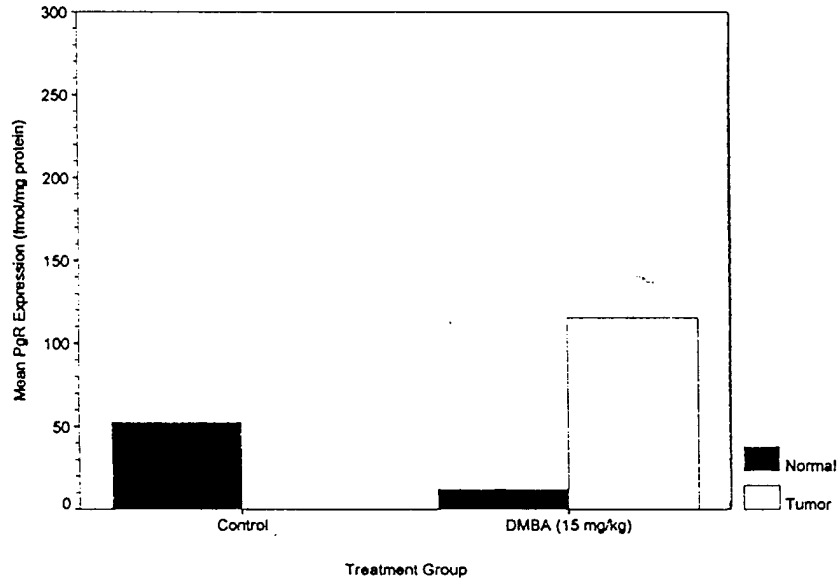

Figure 2C.

Ex#3 Mean PgR Expression in Normal and Neoplastic Mammary Glands

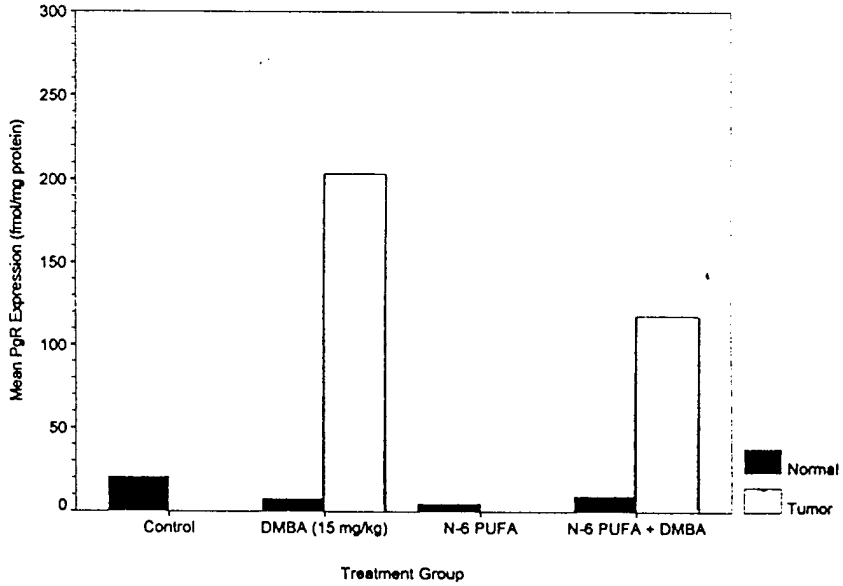

Figure 2. These charts represent the mean PgR levels in grossly normal mammary glands and DMBA-induced mammary gland tumors from *(A)* Experiment #1, *(B)* Experiment #2 and *(C)* Experiment #3. There were significant increases in PgR of DMBA-induced tumors versus grossly normal mammary glands in all three experiments ($p<0.0001$). Control = AIN-76A diet; DMBA = AIN-76A diet + DMBA; N-6 PUFA = high N-6-PUFA diet; N-6 PUFA + DMBA = high N-6 PUFA diet + DMBA

* PgR = Progesterone Receptor (PR)

1B) compared to normal mammary glands in Experiments #1 and 3 ($p<0.001$); however, no significant difference was seen between mammary gland tumors and normal mammary glands in Experiment #2 (Fig. 1C). PR expression was significantly increased in DMBA-induced mammary gland tumors compared to normal mammary glands in all three experiments ($p<0.001$) (Fig. 2A, 2B and 2C). In malignant tumors from rats fed the high N-6-PUFA diet in Experiment #3, there was a significant decrease in PR expression ($p=0.001$) compared to tumors in rats fed the control AIN-76A diet (Fig. 2C). There was a reduction in ER in the same tumors, but the difference was not statistically significant (Fig. 1C). These results are summarized in Table 1.

The appearance of increased levels of ER and PR did not precede tumor formation, as grossly normal mammary glands showed no significant change with DMBA or the high N-6-PUFA diet in ER or PR at any time point studied between 6 hours and 17 weeks after DMBA administration (Fig. 3). Uteri from rats studied 9 and 17 weeks post-DMBA showed no statistically significant effect of DMBA or diet on ER or PR; there was a non-significant decrease in ER and a concomitant increase in PR with age in all groups (data not shown). These results were consistent with previous studies of the rat uterine receptors in Dr. Traish's laboratory (unpublished observations).

The histoscore (H-score) analysis of ER immunohistochemical staining of tumors from rats in Experiment #1 was consistent with the biochemical data showing a significant increase over normal mammary glands ($p=0.02$) (Table 2). This was further supported by quantitative image analysis (data not shown). This evidence supports the biochemical data as a true measure of the epithelial and tumor ER component. A sample of tumors was reanalyzed to evaluate the contribution of ER in the stromal cells. No stromal cell staining was seen, indicating that the biochemical data measured only the epithelial cell ER. In contrast to the ER results, analysis of the PR immunohistochemical staining of normal mammary glands and mammary gland tumors in Experiment #1 showed no significant difference in PR levels (Table 2) between normal mammary glands and tumors. Experiment #4 normal mammary glands showed no significant change in PR H-scores between groups with age (not shown). The data from tumors and normal mammary glands in rats in Experiment #1 showed a non-significant increase in H-scores of PCNA levels in tumors (Table 2), and similar data were obtained with the normal mammary glands in Experiment #4 (not shown).

Thus, DMBA-induced mammary gland tumors have significantly increased PR expression compared to normal mammary glands in all experiments. ER expression in DMBA-induced mammary gland tumors was significantly increased compared to normal mammary glands in Experiments #1 and 3; however, no significant difference was seen between tumors and normal glands in Experiment #2. Tumors in rats fed the high N-6 PUFA diet expressed significantly lower PR than tumors in rats fed control diet; the diet had no effect on ER expression in tumors. DMBA did not change ER expression when measured at 6 hours, 24 hours, 1 week, 3 weeks, 9 weeks or 17 weeks after exposure in normal mammary glands. DMBA appeared to influence PR expression in normal mammary glands in rats 16-17 weeks after exposure, but the results were not consistent among experiments, possibly because different doses of DMBA were used. PCNA measurement of cell proliferation showed no significant difference between tumor and normal mammary glands and no effect of DMBA. In all the experiments, the strain and age of the rats were the same, as was the purified control AIN-76A diet they ate. Therefore these factors can not explain the variation in these results in ER expression. The different doses of DMBA could be contributing factors to these results; however, the randomness observed across experiments could also be attributed to the natural variability seen in an out-bred rat population. Further experiments will be required to distinguish between these possibilities.

Table 1

Estrogen and Progesterone Receptors in Normal Mammary Glands and Mammary Gland Tumors from Control and DMBA-Treated Female Sprague-Dawley Rats (Experiments #1, 2 and 3)

		Experiment #1			Experiment #2			Experiment #3		
		n*	ER**	PR**	n*	ER**	PR**	n*	ER**	PgR**
Control Diet:										
Normal glands										
	Control	7	10±16	6±9	5	24±22	53±53	5	13±12	20±9
	DMBA	50	9±12	26±28[a]	80	10±27	12±18[b]	19	7±7	8±7
Tumor		63	36±25	257±159	39	14±14	136±126	6	27±7	275±81
High N-6 PUFA Diet:										
Normal glands										
	Control							5	14±5	5±10
	DMBA							30	7±8	9±8
Tumor								52	22±11	119±73[c]

* number of samples assayed

** expressed as mean fmol/mg protein ± S.D.

ER = estrogen receptor, PR = progesterone receptor, N-6 PUFA = N-6 polyunsaturated fatty acid

[a] p=0.07 PR in DMBA-treated normal glands increased compared to untreated normal glands

[b] p<0.001 PR in DMBA-treated normal glands significantly decreased compared to untreated normal glands

[c] p<0.001 PR in tumors from HF diet-fed rats significantly decreased compared to tumors from control diet-fed rats

Figure 3A.

Mean ER Expression in Normal Mammary Glands 6 hrs - 17 wks after DMBA

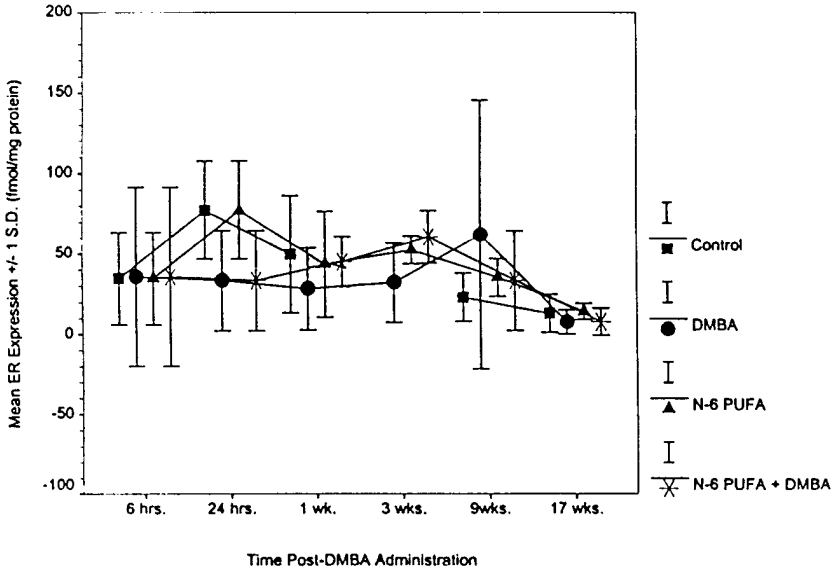

Figure 3B.

Mean PgR Expression in Normal Mammary Glands 6 hrs - 17 wks after DMBA *

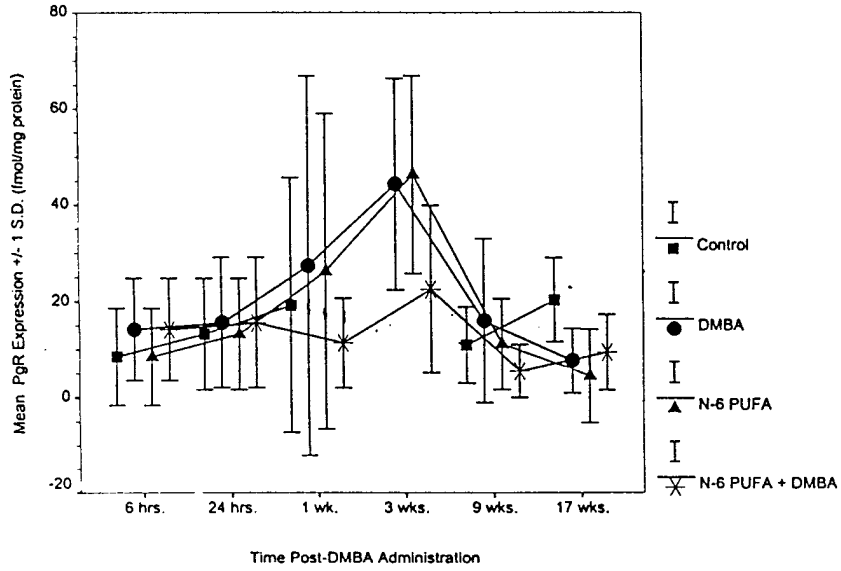

Figure 3. These graphs represent the mean *(A)* ER and *(B)* PgR levels ± standard deviation in normal mammary glands with age (6 hours to 17 weeks post-DMBA). There were no significant changes seen in ER or PgR in the normal mammary glands with age related to DMBA or the high N-6 PUFA diet. Control = AIN-76A diet; DMBA = AIN-76A diet + DMBA; N-6 PUFA = high N-6-PUFA diet; N-6 PUFA + DMBA = high N-6 PUFA diet + DMBA

* PgR = Progesterone Receptor = (PR)

Table 2

Estrogen Receptor, Progesterone Receptor and Cell Proliferation (PCNA) Histoscores in Experiment #1 Normal Mammary Glands and Mammary Gland Tumors

Group	n	ER*	PR*	PCNA*
Normal glands	3	8±14	133±153	112±82
Tumors	25	105±66	133±89	168±91
		(p=0.02)[a]		

* mean histoscore ± 1 S.D.

[a] ER in tumors is significantly increased compared to normal glands

H-score = (Intensity + 1) x Extent

3. DMBA-induced tumors display significant levels of overexpression of AhR RNA and protein, and activation precedes tumor formation.

AhR protein and mRNA levels and CYP1 mRNA expression were evaluated in an rat mammary tumors induced by oral gavage with 15 or 25 mg/kg DMBA as compared with normal mammary glands (using rats from all of the experiments). AhR-specific immunoblotting and immunohistochemistry demonstrated modest levels of AhR protein localized to myoepithelial cells and, to a lesser extent, ductal epithelial cells in normal adult rat mammary tissue. In contrast, high levels of AhR were detected in DMBA-induced fibroadenomas, papillomas, and adenocarcinomas. Specifically, high AhR levels were observed in 88% of invasive cribiform and papillary adenocarcinomas, 72% of non-invasive malignant cribiform and papillary adenocarcinomas and 45% of pre-malignant papillomas, adenomas and fibroadenomas. AhR hyper-expression characterized both tumor cells and stromal cells. Nuclear AhR localization in tumors was suggestive of constitutive AhR activation. *In situ* hybridization studies were consistent with high level *AhR* mRNA expression in the stroma and in neoplastic epithelial cells. Quantitative RT-PCR assays indicated a 27.6-fold increase in *AhR* mRNA in neoplastic as compared with normal rat mammary tissue. While both AhR-regulated *CYP1A1* and *CYP1B1* mRNAs were significantly induced in rat breast tissue within 6 hours of DMBA gavage, only *CYP1B1* mRNA was elevated in tumors 16-18 weeks after DMBA exposure. Collectively, these results: 1) help explain targeting of breast tissue by carcinogenic PAH, 2) imply that AhR and CYP1B1 hyper-expression represent molecular biomarkers for, at least, PAH-induced mammary cell transformation, and 3) suggest mechanisms through which the AhR and AhR-regulated gene products may contribute to carcinogenesis well after exogenous AhR ligands have been eliminated. A copy of a manuscript has been submitted on this work (Trombino et al.), a copy is attached in the Appendix material.

4. Activation of NF-κB precedes tumor formation.

NF-κB/Rel is a family of transcription factors, which are expressed in all cells; however, in most non-B cells, they are sequestered in the cytoplasm in inactive complexes with specific inhibitory proteins, termed IκBs. This inactive NF-κB can be induced by treatment with several agents, including oxidative stress. Our interest in NF-κB relates to our findings that it is a primary regulator of c-*myc* gene transcription. In particular, in our original application we hypothesized a novel mechanism of AhR action. Specifically we proposed that the induction of P-450 enzymes following AhR administration could lead to oxidative stress and thereby to induction of NF-κB. Under the support of this grant, we have shown that NF-κB/Rel factors are aberrantly activated in the mammary tumors induced upon carcinogen treatment of Sprague-Dawley (S-D) rats. Normal rat mammary glands contain the expected low basal levels. We further demonstrated that the aberrant NF-κB/Rel activity functions to promote human breast tumor cell survival *in vitro*. These data have been published: Sovak et al., J. Clinical Investigation 100: 2952-2960 (1997). A copy of this paper is included in the Appendix.

We next sought to determine whether NF-κB/Rel activation occurs prior to tumor formation in the S-D Rat DMBA-model.. The S-D rats treated with DMBA were characterized at the following time points: 6 and 24 hours, and 1, 3 or 9 weeks (Experiment 4). To facilitate the analysis, it was decided to pool all 12 glands for each animal, except for two nine week animals, where non-palpable tumors were found in one gland. These tumors were excluded from the remaining mammary glands samples, which were grossly normal in appearance with no sign of malignant transformation. An aliquot of a nuclear extract from tumor 2-118 L2 was added to each gel to allow for comparison between gels. Autoradiographs from multiple exposures of the

gels were subjected to densitometric analysis and values given as percent of that seen in the tumor 2-118L2 (T) lane. Levels in mammary glands from control, untreated rats varied between 2 and 10% of the value seen in the tumor lane; no significant elevation in binding was seen after 6 or 24 hours or 1 week of DMBA treatment (data not shown). In contrast after 3 weeks, 2 of 5 animals displayed a statistically significant elevation in binding, i.e. above two standard deviations from the mean (Fig. 4). Thus, activation of NF-κB/Rel occurs prior to tumor formation. Similarly, elevated NF-κB/Rel expression was detected in 2 of the 5 animals (40%) 9 weeks following DMBA administration (data not shown). Intriguingly, these were the same animals that had developed tumors. Lastly, it should be noted that if activation had occurred in only a single gland, the pooling of the glands may have masked the full extent of the induction. Thus, we can conclude that NF-κB/Rel activation is an early event in carcinogen-mediated transformation, occurring prior to malignant transformation.

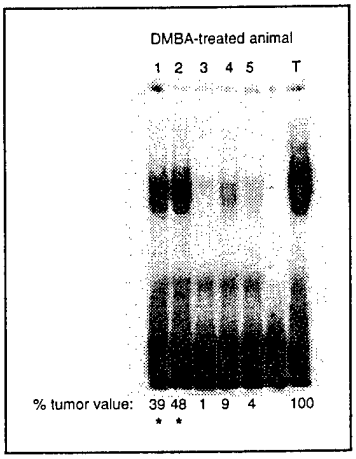

Fig. 4. Activation of NF-κB/Rel is seen after 3 weeks of DMBA treatment of rats. Nuclear extracts were prepared 3 weeks post DMBA administration from rats 1-5. NF-κB/Rel binding to URE was compared to that of 2-118 L2 tumor (T). Numbers under each lane represent the percent value of the intensity of that sample, as measured by densitometry, compared to the intensity of the tumor nuclear extract, which was set at 100%. Samples which have levels of binding at least two standard deviation above the average control samples are indicated by an asterisk.

5. High fat diet induces NF-κB/Rel activity in rat mammary glands.

Although it is clear that a high fat diet increases breast cancer formation in rats, the molecular basis of this observation is unknown. Thus, we examined the effects of a high fat diet on NF-κB/Rel expression in rat mammary tissue (Experiment 3). Nuclear extracts prepared from combined mammary gland preparations for each animal, and used in EMSA. To be able to compare with our previous findings, nuclear extracts were included from two non-DMBA treated animals on a control diet to see the range of normal (4-1, low vs 4-7 high). The intensity of binding with nuclear extracts from 3 out of 5 animals fed a high fat diet was clearly above these basal levels (Fig. 5). In the sample of five animals administered DMBA and fed a high fat diet, 2 rats (4-116 and 4-119) showed elevated levels of binding. As expected, none of the rats had developed tumors by 3 weeks. These preliminary experiments indicate that a high fat diet alone can aberrantly induce NF-κB/Rel binding activity after 3 weeks.

6. Expression of ER and PR in older breast cancer patients.

We have examined the expression of estrogen (ER) and progesterone receptors (PR) and the distribution of tumor phenotypes as a function of age in breast cancer patients. ER and PR concentrations were determined in tissue biopsies from 1739 specimens of patients with primary breast cancer, using ligand-binding assays. Tumors were classified as receptor positive (ER+) or

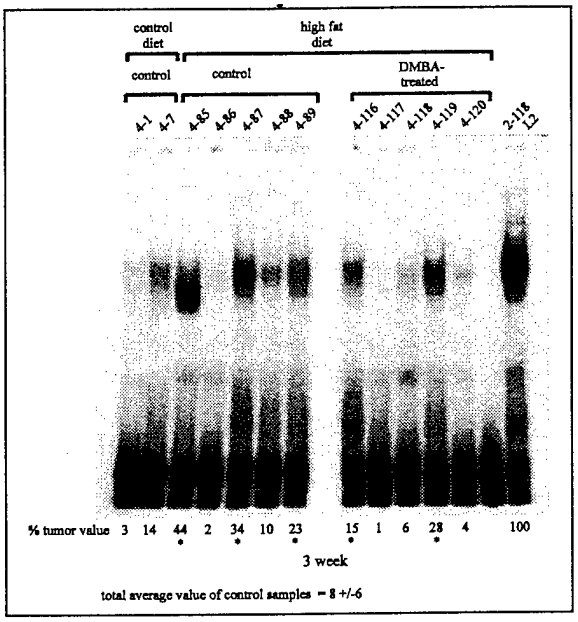

Fig. 5. EMSA analysis on the nuclear extracts isolated from mammary tissue from control and DMBA- treated rats on a high fat diet 3 weeks after carcinogen treatment. After 4 days, DMBA-administered and control rats were switched to a high fat diet. Animals were sacrificed after 3 weeks and nuclear extracts prepared from combined mammary gland preparations from each animal. NF-κB/Rel binding levels were assessed using the URE oligonucleotide as probe. Rats 4-1 and 4-7 from untreated animals (normal diet, no DMBA treatment) were included to represent the range of binding levels detected in control mammary tissue. *, significant level of increase compared to average control

negative (ER-) and PR+ or PR- based on the presence or absence of receptor binding activity. Tumors were stratified into four phenotypes based on the combined ER and PR status: ER+ PR+; ER+ PR-; ER- PR+; and ER- PR-. Significant positive associations were found between ER and age (p=0.0001) and between PR and age (p=0.0002). The median ER levels were statistically different age groups, with the greatest levels older versus younger patients. The prevalence of ER+PR+ tumor phenotype increased with age. In contrast, the prevalence of ER-PR- and ER-PR+ tumor phenotypes decreased with age. The median PR to ER ratio decreased with age (p=0.0001) and this trend was attributed to increased ER levels with age. The prevalence of ER-PR- and ER-PR+ tumor phenotypes in younger patients suggest that hormonal regulation of ER gene expression may be responsible for the observed disparity of tumor phenotypes in breast cancer.

The increased prevalence of ER+PR+ tumor phenotype with age may be attributed to up-regulation of ER expression by decreased circulating levels of endocrine hormones. The decrease in the prevalence of ER- PR- and ER- PR+ tumor phenotypes with age may represent a different mechanism of regulation of ER expression when endocrine hormones are altered with age. Thus, the progression of tumor phenotype from ER+ PR+ to ER- PR- may be the result of tumor cell de-differentiation in response to other endocrine factors or a manifestation of tumor progression to acquire growth advantages, becoming less dependent on estrogens. Fine needle aspirate studies of normal and cancerous breast tissue in pre-menopausal women have shown that in normal subjects, ER synthesis is cyclic and is mainly produced when plasma estrogen and progesterone concentrations are reduced (during the early follicular phase). In contrast to normal breast tissue, in older patients, ER expression in breast cancer tissue may occur constitutively. Hence, among pre-menopausal patients, information on the menstrual cycle stage at the time of biopsy may be important in understanding the possible effects on tumor hormonal sensitivity.

The discordant tumors (ER+PR- and ER-PR+) are not clearly understood. Tumors with ER-PR+ phenotype were found mostly in the youngest age group (<45 years), and the prevalence decreased with age. This may be explained by other factors, such as the use of oral

contraceptives, or by cyclic changes in plasma estradiol and progesterone levels in pre-menopausal women. Our findings are limited because of the lack of information regarding other possible cofounders on the association between age and ER.

The presence of tumor phenotypes with ER+ PR- may represent the expression of non-functional ER in such tumors. PR expression is dependent on ER function, thus lack of expression of PR is attributed to the presence of ER, which has the ability to bind the hormone but cannot elicit a biological function. Similarly, the presence of the ER- PR+ tumor phenotype may represent the expression of ER with an altered hormone binding domain which remains biologically active. Indeed such phenotypes have been described. The prevalence of ER+ PR- tumors did not exhibit any consistent trend with age. One explanation may be that ER+PR- tumors represent a transition phase in cell differentiation or a stage in the growth of breast cancer. These tumors may also characterize the population of breast cancer patients that do not respond to endocrine therapy due to the presence of dysfunctional ER. Since there are significant differences in the prevalence of tumor phenotype among the various age groups, it is likely that lack of ER expression or function is associated with disease progression and tumor phenotypic changes with age. We were unable to correlate these findings with other breast cancer risk factors (e.g. family history, age at menarche, parity) because such data were inaccessible.

The discrepancies in the prevalence of tumor phenotypes between our study and others may be attributed to limitations of receptor assay techniques, choice of different cut-off values for ER and PR status, or the age distribution of the population studied. Further, other factors such as tumor tissue procurement and handling, assay conditions, previous radiotherapy treatment prior to surgery or treatment with anti-estrogens prior to surgery may affect ER and PR values. Our study confirms and extends the observations made in previous reports in which ER+PR+ tumors were shown to be most prevalent in older age groups. However, our study shows that ER-PR- tumors were less prevalent with age, contrary to other reports. Additional prospective studies are needed to address the question of whether the four tumor phenotypes represent multiple forms of cancer or different stages in the de-differentiation process of one form of cancer. These studies have been reported (Ashba and Traish, 1999; cf enclosed manuscript).

SPECIFIC AIM 2: ALTERATIONS IN STRUCTURE AND FUNCTION OF C-MYC AND ER

Mutations in c-*myc* have often been found to result in altered function, in particular, decreased ability to repress initiator element (Inr)-mediated gene transcription. Recently we have identified a novel target of c-Myc repression, the p27Kip1 cyclin dependent kinase inhibitor, which mediates control of progression through G1 and entry into the S-phase. Thus, we have explored the ability of c-Myc to repress the p27 promoter in transient transfection and found an approximate 5-fold repression in Hs578T cells. This level of repression is comparable with that found in other normal and transformed cells in the laboratory. This finding suggests that c-Myc-mediated repression is operative in the breast cancer cells. Thus, our exploratory experiments suggests that c-Myc is not altered in these breast cancers. Interestingly, our findings are consistent with recent results by several groups that show that the expression of p27 is extremely low in breast cancer cells while c-Myc expression is high. Interestingly, the drop in p27 expression has been found to correlate with poor prognosis, as does the increase in c-Myc.

1. Studies on Estrogen Receptor Activation and Phosphorylation by Hydroxylated Chlorobiphenyls.

Estrogen receptor activation is a critical step in in mediating receptor function. Phosphorylation of ER at Ser 119 is shown to be critical to ER activation. We have developed site-directed, monoclonal antibodies to ER which detect both phosphorylated and non-phosphorylated receptor forms. We have developed an assay, which distinguishes between these two forms. We have utilized this new assay to assess the potential estrogenic activity of chlorobiphenyls, a group of chemicals suspected of having estrogenic activity and may possess endocrine disruptive effects. Treatment of MCF-7 cells in culture with estradiol results in phosphorylation of ER, as determined by western blot analysis using specific site-directed monoclonal antibodies to ER. The phosphorylation of ER correlates with receptor activation *in situ.* Several hydroxylated polychlorinated biphenyls were tested for their ability to bind to ER and to activate ER. We have found that hydroxylated chlorobiphenyls displace estradiol from the receptor competitively and with moderate affinity and induce ER activation, as demonstrated by ER phosphorylation. This new assay system provides new and simple assay for screening of chemical compounds with potential estrogenic activity. A preliminary account of these observations has been reported (abstract enclosed). We have also tested the potential estrogenic activity of dietary or phytoestrogens in activating ER. We have found that substances in tea extracts are capable of activating ER in intact cells and binding to ER in tissue extracts. These observations suggest that this new assay is suitable for testing environmental and phytoestrogenic compounds in a simple and rapid assay *in situ.* A preliminary account of these observations has been reported (abstract enclosed).

2. Studies on characterization of a novel nuclear matrix protein in human breast cancer.

a) Relationship to tumor hormonal status. The development of breast cancer is thought to be a multi-stage process. The progression of this disease is associated with cellular and molecular changes. Thus, initiation and progression may be associated with loss of chromosomal material and ultimately specific gene function (s). Some of these cellular and molecular changes may be associated with tumor cell acquisition of metastatic potential. There is an urgent need for identification of node-negative patients whose tumors have a metastatic potential. Several tumor markers have been used in assessing tumor changes linked to poor prognosis. These include loss of ER and PR, high blood vessel count (angiogenesis), amplification of *erbB2/HER2/neu* gene and decreased activity of *nm23* gene. None of these markers alone, however, predict, with complete reliability, which node-negative patients will be likely to relapse.

In our search for new ER-related markers, we have identified and characterized a 55 kDa nuclear protein (referred to as *nmt55*) from human breast tumors and MCF-7 cell line, using site-directed monoclonal antibodies to the ER. Measurements of estrogen (ER) and progesterone (PR) receptors, by ligand binding assays, in cytosols of 63 human breast tumors permitted classifications of these tumors into four phenotypes (ER+/PR+, ER+/PR-, ER-/PR-, ER-/PR+). *nmt55* protein expression in these tumors, as determined from Western blot analyses, showed a statistically significant association ($p=0.001$) with tumor hormonal phenotype. A review of the pathological characteristics of tumors analyzed suggested that lack of *nmt55* expression was significantly associated with mean tumor size ($p < 0.03$), mean ER ($p= 0.001$) and mean PR ($p <0.002$), but was not associated with tumor stage, grade or type. To further study this protein, we cloned and sequenced a 2.5-kb cDNA. The complete predicted open reading frame encodes a predicted protein with 471 amino acids and a calculated molecular mass of 54,169 Da. These data area consistent with molecular mass obtained by gel electrophoresis. The deduced amino acid sequence exhibited unique regions rich in glutamine, histidine, arginine, and glutamic acid.

Northern blot analysis of RNA from MCF-7 cells and ER+/PR+ human breast tumors showed a 2.6 kb mRNA. Southern blot analysis suggested that presence of a single copy of this gene. Chromosomal mapping, using fluorescent in situ hybridization (FISH), located *nmt55* gene to the X chromosome, region q13. The extensive homology between *nmt55* and RNA binding proteins suggested that *nmt55* may be involved in hnRNA splicing. The strong association observed between expression of *nmt55*, tumor hormonal phenotype, mean tumor size, mean ER, and mean PR content suggests that loss of *nmt55* expression may be related to events involved in hormone-insensitivity, tumor differentiation and unregulated tumor cell growth and metastases (Traish et al., 1997, enclosed publication).

To investigate the possible regulatory role of nmt55 in cellular function, we have used gel shift mobility assays to determine the putative DNA binding elements for this protein. Using homology sequences with other nuclear proteins, we have shown that nmt55 binds to intercisternal A-particle proximal enhancer element (IPE), with specific DNA sequence, ATCATCAGGGAGTGACACGTCCGA. A preliminary account of these studies has been reported (Schmitt, H. Moreland RB, Savelyeva N ., Hafer L. and Traish AM. Nmt55 binds to intracisternal A-particles proximal enhancer element (IPE) and polypyrimidine tract-splicing factor (PSF). FASEB J. 12: A472 (abstract # 2742) 1998).

b) nmt55 associated with human splicing factor (PSF). To determine if nmt55 associates with members of RNA splicing proteins, we carried out experiments in which cells were labeled in situ with ^{35}S-methionine, extracted and the extracts were immunprecipitated with monoclonal and polyclonal antibodies. Incubation with pre-immune serum did not show a strong band at molecular weights of 55 and 100 kDa, while immunoprecipitation with antibody NMT5 showed a strongly labeled protein band at 100 and 55 kDa, respectively. This suggests that nmt55 associates with a 100 kDa protein. Similarly, immunoprecipitation with pre-immune serum or with antibody NMT4 provided similar results. Using unrelated antibody (ER-213) raised against ER, only a weakly labeled band was detected. In contrast, immunoprecipitation with NMT1 antibody showed strongly labeled bands at 100 and 55 kDa, respectively. To confirm this finding, parallel samples were immunoprecipitated with NMT1 monoclonal antibodies, electrophoresed and electrotransfered and the proteins were detected using specific antibodies to either nmt55 or PSF. Antibodies raised against PSF detected a specific 100 kDa band in the co-immunoprecipitate, suggesting that nmt55 is associated with PSF. Immunoprecipitation with antibodies to PSF also resulted in detection of nmt55 protein in the co-immunoprecipitate. These observations indicate that nmt55 associates with polypyrimidine tract binding protein/splicing factor (PSF).

SPECIFIC AIM 3: TO ELUCIDATE MECHANISMS LEADING TO INCREASED EXPRESSION OF C-*MYC* RNA.

Our earlier work indicated that c-*myc* mRNA levels were increased DMBA-induced tumors as compared with normal mammary gland (as evidenced by *in situ* hybridization; data provided in an earlier report). Using transfection analysis with the c-*myc* promoter, rather than our originally proposed method of run-on analysis, we found that an increased rate of transcription was at least in part responsible for the elevated c-*myc* expression (Task 1; see Sovak et al., 1997 in Appendix). Furthermore, our studies implicated the NF-κB/Rel family of transcription factors as a major player in the transcriptional activation of the c-*myc* gene (Task 2). Therefore, we have

pursued these observations in several directions. We have explored the role of NF-κB in controlling the ability of TGF-β1 to growth arrest breast cancer cells. This factor has been shown to decrease c-*myc* expression. Secondly, we have examined the mechanism of NF-κB activation and found carcinogens induce NF-κB. Lastly, we have obtained evidence for cooperation between the p65 subunit of NF-κB with the AhR receptor/transcription factor in inducing transcription of the c-*myc* gene. Thus, identifying a novel transcription factor complex that may play a role in the observed overexpression of this oncogene in breast cancer cells.

1. The Inhibitory Effects of TGF-β1 on Breast Cancer Cell Proliferation are Mediated through Regulation of Aberrant NF-κB/Rel Expression.

Since we have recently shown that NF-κB/Rel factors are involved in mediating TGF-β1 signals inhibiting growth of hepatocytes, here we analyzed their potential role in the ability of TGF-β1 to inhibit growth of breast cancer cells. Decreased growth of Hs578T and MCF7 breast cancer cell lines upon TGF-β1 treatment correlated with a drop in NF-κB/Rel binding. This decrease was due to stabilization of the inhibitory protein IκB-α. Ectopic expression of c-Rel in Hs578T cells led to maintenance of NF-κB/Rel binding and resistance to TGF-β1-mediated inhibition of proliferation. Similarly, expression of the p65 subunit ablated the inhibition of Hs578T cell growth mediated by TGF-β1. Thus, inhibition of the aberrantly activated, constitutive NF-κB/Rel plays an important role in arrest of proliferation of breast cancer cells, suggesting NF-κB/Rel may be a useful target in the treatment of breast cancer. (A manuscript has recently been accepted on this work (Sovak et al. Cell Growth and Differentiation), a copy is enclosed.)

2. In Vitro carcinogen transformation induces NF-κB/Rel activity.

D3-1 and BP-1 cell lines were derived from untransformed MCF-10F cells by 24 hour treatment of either DMBA and BaP, respectively (103). Both cell lines exhibit the malignant characteristics of increased anchorage independent growth, increased chemotaxis and chemoinvasiveness. D3-1 cells exhibit thse capabilities to a lesser extent than BP-1 cells (103). Nuclear extracts from D3-1 and BP-1 cells displayed significantly increased NF-κB binding activity compared to the parental MCF-10F cells (inset Fig. 6). Two bands were seen with the extracts from the D3-1 and BP-1, which co-migrated with bands seen with nuclear extracts from the MCF-10F cells. (The upper band with MCF-10F extract was better seen on a longer exposure.) To verify functional activity, transient transfection analysis was performed with the wt E8 and dm E8 NF-κB-TK-CAT constructs and SV40-βgal to normalize for transfection efficiency. The data are presented as the activity of wild type construct relative to that of the dm E8, to obviate differences in transfection efficiencies amongst the lines (Fig. 6). The parental MCF-10F cells showed a minimal induction of wt E8 activity over the dm E8 ranging from 1.7-fold +/- 0.6. The D3-1 and BP-1 cells showed an induction of NF-κB activity of 4.1-fold +/- 1.4 and 11.6-fold +/- 0.2, respectively. Thus, the relative levels of binding and activity correlate directly. Supershift analysis, indicated the upper and lower complexes are composed of classical NF-κB (p50/p65) and p50 homodimers, respectively (data not shown). Thus, the chemically transformed D3-1 and BP-1 cell lines display increased constitutive levels of functional classical NF-κB than seen in the parental MCF-10F cells.

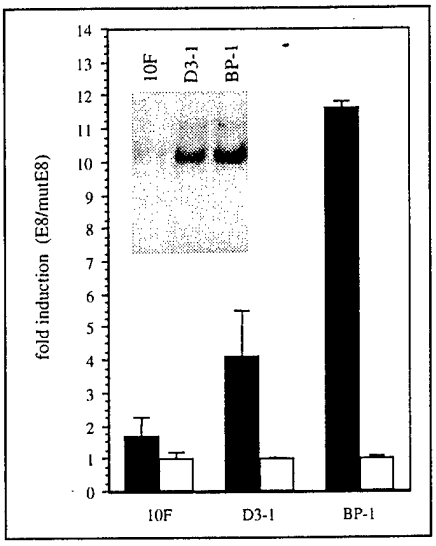

Fig. 6. Carcinogen-transformed D3-1 and BP-1 cells display higher constitutive levels of functional NF-κB than the parental MCF-10F cells. The MCF-10F cells (10F) and BP-1 cells were transiently transfected by lipofection, in triplicate or duplicate, respectively with 2 ug wt E8 or dm E8 reporter construct. D3-1 cells were transfected, in duplicate, using 20ug of the same vectors by the calcium phosphate method. After 24 hours (for lipofectamine) or 72 hours (for calcium phosphate), extracts were prepared, normalized for protein, and assayed for CAT activity. The values for wt E8 CAT activity are represented as fold induction over dm E8 CAT activity which was set at 1.0 for each cell line. (Inset) Equal amounts (5 ug) of nuclear extracts from exponentially growing parental MCF-10F cells or transformed D3-1 or BP-1 cells were subjected to EMSA with a radiolabelled URE NF-κB oligonucleotide as probe.

3. Physical Interaction between AhR and the p65 NF-κB Subunit Enhances Transcriptional Activation of the c-myc Promoter.

Recently, a physical association was demonstrated between the aromatic hydrocarbon receptor/transcription factor (AhR) and the p65 (RelA) subunit of NF-κB by Tian and coworkers in hepatocytes (104), and confirmed by David Sherr's laboratory in breast epithelial cells (data not shown). These observations led us to test whether the two factors could cooperate in transactivation. Transient transfection analysis was performed into Hs578T cells using the c-*myc* promoter-CAT reporter (p1.6 Bgl) which contains two NF-κB elements. In our initial analysis, expression of the AhR led to a slight increase in activity of the c-*myc* promoter (Fig. 7). We then determined the suboptimal doses of p65 that when added alone would not affect c-*myc* promoter activity (0.25 ug DNA). When Hs578T cells were co-transfected with the p65 and AhR expression vectors, in two separate experiments, a reproducible increase in CAT activity was seen compared to AhR alone (Fig. 7).

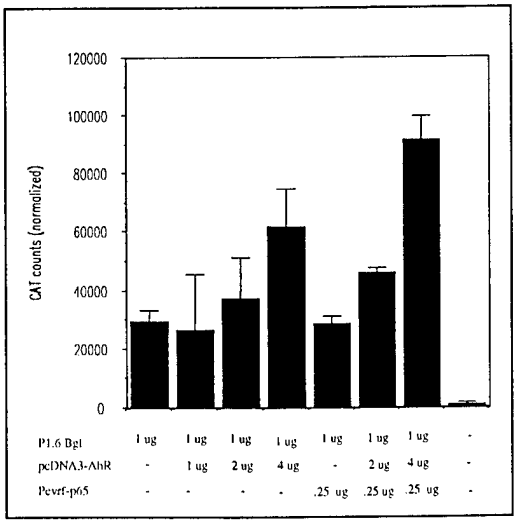

Fig. 7. AhR and p65 cooperate to transactivate the c-*myc* promoter in transient transfection analysis in Hs578T cells. Confluent cultures of Hs578T breast cancer cells were transfected in duplicate using FUGENE reagent (Roche) with 1 ug p1.6Bgl c-myc-CAT DNA in the absence or presence of 1, 2, or 4 ug AhR (pcDNA3-AhR) expression vector or 0.25 ug p65 expression (pEVRF-65), as indicated. The p1.6 Bgl c-myc-CAT contains 1.2 kb of upstream region plus 0.4 kb exon1 of the murine c-*myc* gene, including the upstream and internal NF-κB elements, driving the CAT reporter. The total amount of DNA was adjusted to 5.5 ug with pcDNA3 empty vector DNA. Cells were harvested after 24 hours and equal amounts of proteins analyzed for CAT activity.

Since the Hs578T cells express significant levels of functional p65 without transfection, we next repeated the transfection analysis with MCF-10F cells, which we showed contain much lower levels of nuclear NF-κB. As can be seen in Figure 8, a very significant increase in c-*myc* activity was seen upon co-transfection of the AhR and p65 expression vectors compared to either vector alone. To test whether binding of NF-κB was required, a similar co-transfection analysis was performed using a c-*myc* promoter construct mutated at both NF-κB elements (p1.6Bgl double mutant) such that it cannot be effectively transactivated by classical NF-κB. The basal activity of the double mutant construct was reduced only slightly compared to the wild type p1.6 Bgl, consistent with the low level of constitutive NF-κB activity in these cells. The mutant construct, however, failed to be induced by co-transfection with p65 and AhR (Fig. 8). These results suggest that AhR and p65 can synergize to enhance c-*myc* promoter activity in MCF-10F cells and that binding of NF-κB appears required.

We next decided to test for direct binding of p65 and AhR to an NF-κB element from the c-*myc* gene and selected the upstream element (termed URE), which we have shown binds all known mammalian members of the NF-κB/Rel family. Nuclear extracts were prepared from Hs578T cells alone or following transfection with an AhR expression vector either in the absence or presence of a p65 expression vector. (The Hs578T line was selected for these transfection analyses based on the significantly higher level of transfection efficiency achievable with this line compared to the MCF-10F cells.) A novel slower migrating complex was seen upon co-transfection with both constructs (Fig. 9A). This upper band appeared specific based on successful competition with cold wt but not mutant oligonucleotide (Fig. 9B). Furthermore, we could show that an antibody against the AhR protein removed this upper complex as did addition of an antibody against the p65 subunit (Fig. 10). This antibody also blocked formation of a band that we had previously identified as p50/p65, classical NF-κB. As control for antibody specificity, we observed that the AhR antibody does not affect binding to an irrelevant Oct-1 oligonucleotide (Figure 10B), and that addition of an antibody against another transcription factor YY1, does not alter binding (data not shown). Together, these preliminary results indicate a novel complex containing p65 and AhR can bind to NF-κB element sequence and transactivate the c- *myc* promoter.

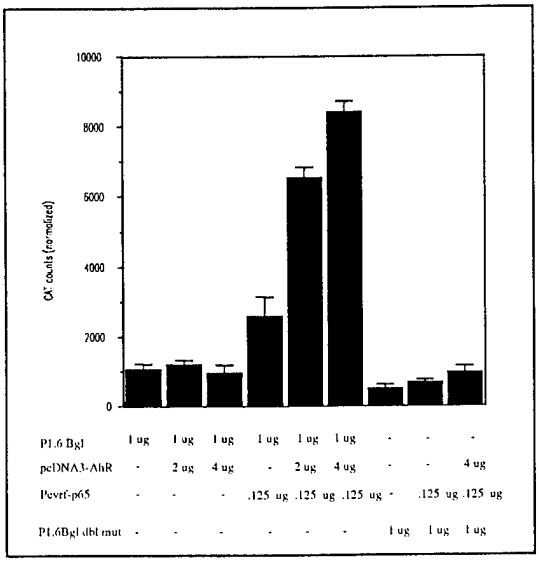

Fig. 8. AhR and p65 synergize to transactivate the c-*myc* promoter in MCF10F cells. Confluent cultures of MCF10F cells were transfected in duplicate using FUGENE with 1 ug p1.6Bgl c-myc-promoter CAT reporter vector, in the absence or presence of 2, or 4 ug AhR expression vector or 0.125 ug p65 expression, as indicated. In addition 1 ug of TK-luciferase was added to each transfection mix to allow for normalization of transfection. The total amount of DNA was adjusted to 6 ug with pcDNA3 empty vector DNA. A double mutant version of p1.6 Bgl c-myc CAT (p1.6 dbl mut), in which the two G to C conversions were introduced into the upstream and internal NF-κB elements, was similarly analyzed. Cells were harvested after 24 hours and lysates normalized for luciferase activity were analyzed for CAT enzyme activity.

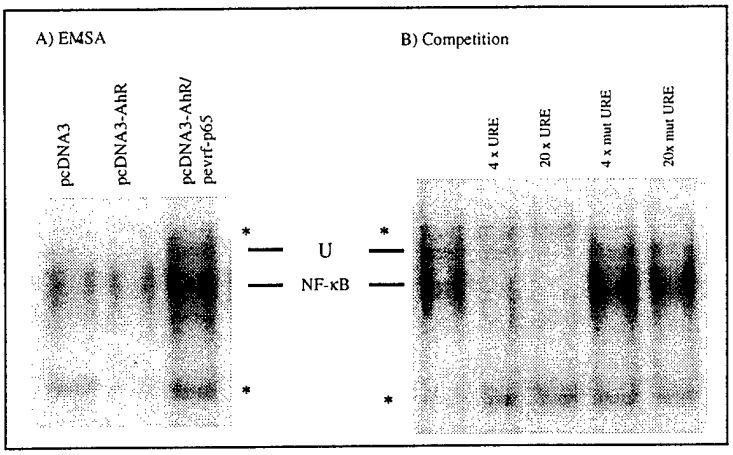

Fig. 9. Co-transfection of Hs578T cells with an AhR and p65 expression vectors leads to the presence of a novel specific NF-κB element binding complex. A) EMSA. Confluent HS578T cells in P100 dishes were transfected with either 50 ug pcDNA3-AhR DNA alone or in the presence of 2 ug p65 expression vector DNA or with 52 ug of pcDNA3 empty vector DNA. After 24 hours, nuclear lysates were prepared and 2.5 ug incubated for 30 minutes at room temperature with ^{32}P-oligonucleotide of the upstream NF-κB element (URE) from the c-*myc* gene, and subjected to EMSA. B) Competition analysis. Lysates from cells transfected with AhR and p65 expression vectors, prepared above, were used in EMSA with the wt URE NF-κB oligonucleotide as probe, in the absence or presence of 4- or 20-fold molar excess unlabelled wild type or double mutant URE as competitor. U, position of new upper complex; NF-κB, complex containing p65/p50; *, non-specific binding complex.

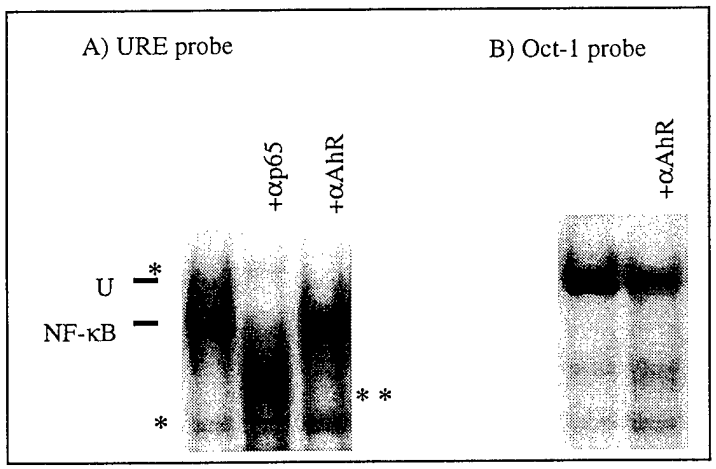

Fig. 10. An AhR- and p65-containing complex binds to the NF-κB upstream element from the c-*myc* gene. Supershift EMSA was performed with the lysates from Hs578T cells transfected with AhR and p65 expression vectors, prepared above. Equal amounts of lysate (2.5 ug) were used in a 30 minute binding reaction, as above, and then incubated for an additional 1 hour at room temperature in the absence or presence of 1 ul of antibody against the p65 or AhR protein, as indicated. A) The upstream NF-κB element (URE) from the c-*myc* gene was used as probe. U, position of upper complex; NF-κB, complex containing p65/p50; *, non-specific binding complex; **, non-specific complex resulting from antibody addition. B) The Oct-1 oligonucleotide probe.

4. Determine AhR function in pre-malignant and malignant human breast cancer cell lines and evaluate AhR expression in primary human mammary tumors.

To evaluate the hypothesis that AhR up-regulation is causally related to proliferative capacity associated with malignancy, we are modulating AhR activity in mammary tumor cell lines using human breast cancer cell lines. *AhR*-specific RT-PCR demonstrated low levels of *AhR* mRNA in normal breast epithelial cells (HS578BST, 184S and 184L) but approximately 10 times higher levels ($p<0.001$) in pre-malignant (184-A1, 184-B5, and MCF-10F) and malignant (CAMA-1, MCF-7, MDA-MB-231, and HS578T) cells. Similarly, western immunoblotting indicated that nuclear AhR levels in human pre-malignant and malignant breast cancer cell lines were 8.2 times higher than those in normal breast epithelial cells ($p<0.004$) (not shown). These results are consistent with those obtained with rat tissue and hint at a role for the AhR at a relatively early stage in cell transformation.

If constitutive AhR activity is causally related to tumor cell growth, then AhR inhibitors should slow cell growth. Three AhR inhibitors, α-naphthoflavone (ANF), galangin, and indole-3-carbinol (I3C) were added to cultures of pre-malignant MCF-10F or 184-A1 cells. Galangin is a naturally occurring, vegetable-derived bioflavonoid which we have recently shown to be a strong AhR antagonist. All three compounds (10^{-5}-10^{-6} M) significantly slowed MCF-10F and 184-A1 cell growth as assayed either by cell recovery after 48 hours in culture or ^{3}H-thymidine incorporation over an 18 hour culture period, without affecting cell viability. FACS analysis of cellular DNA content indicate that AhR antagonist inhibition of cell growth occurs at the G_1-S transition in cell cycle. This growth inhibition is not due to down-regulation of estrogen receptors, as suggested in other systems. These data support a causal relationship between AhR activity and cell growth and suggest novel, AhR-targeted approaches to breast cancer prevention and treatment.

5. Develop and test transgenic mouse in which AhR and NF-κB expression in mammary tissue is altered.

To test the roles of NF-κB and AhR in the mammary gland, we have decided to use a transgenic mouse strategy. For NF-κB, we are taking advantage of the IκB-α protein to inhibit its activity. Furthermore, we are also using ectopic subunit overexpression; for these experiments we have selected the c-Rel subunit. This subunit has the advantage that is a potent transactivator, but appears less susceptible to inhibition by IκB proteins than the p65 subunit. For the AhR, we have begun by assessing the role of elevated AhR in the mammary gland. The LTR of the mouse mammary tumor virus (MMTV) has been selected as a specific promoter to direct cDNA expression to mammary epithelial cells in transgenic mice. We have constructed *MMTV-c-Rel*, *MMTV-IκB-α*, and *MMTV-AhR* transgenes. These DNAs have been injected into FVB mouse oocytes. For each of these constructs, a minimum of 3 potential founder mice have been identified using Southern blot analysis of tail DNA. These are currently being bred and screened for appropriate transgene expression. Once confirmed, the mice will be studied for effects of transgene expression on normal mammary gland development, as well as for tumor formation.

7. KEY RESEARCH ACCOMPLISHMENTS

In the work presented in this application, we have:

A. Demonstrated that NF-κB/Rel is aberrantly activated in breast cancer using rat mammary tumor tissue compared to normal mammary glands.
B. Demonstrated that NF-κB/Rel expression is elevated in cultured human breast cancer cells vs untransformed epithelial cells.
C. Demonstrated aberrant nuclear NF-κB p65, c-Rel, and p50 subunit expression in multiple primary patient tissue using immunoblot analysis.
D. Demonstrated that inhibition of the aberrant NF-κB in breast cancer cells in culture leads to apoptosis, and hence this factor is a potential target for adjuvant therapy.
E. Demonstrated that DMBA-induced tumors displayed significantly increased levels of progesterone receptor.
F. Demonstrated that DMBA-tumors and normal mammary glands had no significant differences in cell proliferation as judged by PCNA measurements.
G. Demonstrated the expression of the AhR is increased in DMBA-induced rat mammary tumors as evidenced by *in situ* hybridization, quantitative RT-PCR and immunoblotting.
H. Demonstrated AhR mRNA and protein levels are increased in human breast cancer specimens.
I. Identified and cloned a novel nuclear matrix protein nmt55 whose expression is reduced in ER- human breast cancer.
J. Shown that selective inhibitors of AhR slow growth of human breast cancer cell lines.
K. Demonstrated c-*myc* mRNA levels are elevated in rat DMBA-induced mammary tumors using *in situ* hybridization.
L. Demonstrated that the inhibitory effects of TGF-β1 on human breast cancer proliferation are mediated through regulation of the aberrant NF-κB/Rel expression.
M. Demonstrated NF-κB levels are increased by PAH exposure in human breast epithelial cells.
N. Identified and characterized a novel mechanism of p65 and AhR interaction that leads to the induction of c-*myc* gene transcription.
O. Prepared transgenic *MMTV-AhR* to study of the role of this factor in development of the normal mammary gland as well as breast cancer.
P. Prepared transgenic *MMTV-c-Rel* and *MMTV-IκB-α* mice for study of the roles of this family of factors in development of the normal mammary gland as well as breast cancer.

8. REPORTABLE OUTCOMES

MANUSCRIPTS, ABSTRACTS AND PRESENTATIONS

Sovak, M.A., R.E. Bellas, D.W. Kim, G.J. Zanieski, A.E. Rogers, A.M. Traish, and G.E. Sonenshein. Aberrant NF-κB/Rel expression and the pathogenesis of breast cancer. J. Clin. Investig. 100, 2952-2960 (1997).

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D.W. Kim, M.A. Sovak, G. Zanieski, G. Nonet, P. Yaswen, M. Stampfer, J. Russo, A.E. Rogers, and G.E. Sonenshein. Activation of NF-κB/Rel occurs early during neoplastic transformation of mammary cells and involves decreased IκB-α stability. (manuscript submitted).

A.F. Trombino, R.I. Near, R.A. Matulka, S. Yang, L.J. Hafer, P.A. Toselli, D.W. Kim, A.E. Rogers, G.E. Sonenshein, and D.H. Sherr. Expression of the Aryl Hydrocarbon Receptor/Transcription Factor (AhR) and AhR-regulated *CYP1* gene transcripts in a rat model of mammary tumorigenesis. (manuscript submitted).

W. Yang, M. Wu, M.J. FitzGerald, R.E. Bellas, J. Shen, D.W. Kim, Z. Suldan, L.P. Freedman, and G.E. Sonenshein. Repression of transcription of the pro-apoptotic $p27^{Kip1}$ cyclin dependent kinase inhibitor gene by c-Myc. (manuscript submitted).

Rogers, A.E., L.M. Sullivan and L.J. Hafer. Dietary fat, body weight and cancer: Contributions of studies in rodents to understanding these cancer risk factors in humans. (manuscript submitted).

D.W. Kim, L. Gazourian, S. Quadri, D.H. Sherr, and G.E. Sonenshein. The Aryl Hydrocarbon Receptor/Transcription Factor (AhR) and the RelA subunit of NF-κB synergize to transactivate the c-*myc* promoter. (manuscript in preparation).

Quadri S, Qadri A, Sherr DH. Galangin, a naturally occuring bioflavonoid is an aryl hydrocarbon receptor/transcription factor inhibitor. (manuscript in preparation).

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Trombino, AF, Yang, S., Hafer L.J., Qadri, A.N., Rogers, A.E. and Sherr, D.H.. Modulation of aromatic hydrocarbon receptor expression in 7,12-dimethylbenz[*a*]anthracene-induced rat mammary tumors. Society of Toxicology Annual Meeting, Anaheim, CA. April, 1997. The Toxicologist.

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Rogers AE, LJ Hafer, YS Iskander and S Yang. Mammary Gland Carcinogenesis, Black Tea and Dietary Fat", Toxicological Sciences, Abstract #1558, 42(1-S): 317, 1998.

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POSTER PRESENTATIONS

Hafer LJ, YS Iskander, S Marecki and AE Rogers, "Black Tea and Mammary Carcinogenesis in Rats", 1996 American Association For Cancer Research Workshop -- Histopathobiology of Neoplasia (Keystone, CO).

Trombino AF, S Yang, LJ Hafer , AN Qadri, AE Rogers and DH Sherr, "Modulation of Aromatic Hydrocarbon Receptor Expression in 7,12-Dimethybenz(*a*)anthracene-induced Rat Mammary Tumors", 1997 Henry I Russek Student Achievement Day (Boston, MA).

Traish AM, K Murphy, L Hafer, N Savelyeva and AE Rogers, "Tea Polyphenols Bind to Estrogen Receptor and Induce Its Phosphorylation", 80th Annual Meeting of the Endocrine Society, Abstract #P1-567, June 24-27, 1998.

Hafer LJ, KE Murphy, AE Rogers, AM Traish, YS Iskander, S Yang and PF Johansen, "Black Tea, High Dietary Fat and Steroid Hormone Receptors in Mammary Gland Carcinogenesis in Sprague-Dawley Rats", 1998 Henry I Russek Student Achievement Day (Boston, MA).

Hafer LJ, KE Murphy, AE Rogers, AM Traish and PF Johansen, "Black Tea and Steroid Hormone Receptors in Normal and Neoplastic Mammary Glands in Rats", 1998 Society of Toxicology Annual Meeting (Seattle, WA).

D.W. Kim, M.A. Sovak, G. Zanieski, G. Nonet, P. Yaswen, M. Stampfer, J. Russo, A.E. Rogers, and G.E. Sonenshein. Mammary Gland Biology Gordon Conference May 1999, Poster presentation: "NF-κB in Breast Cancer: Aberrant Activation Occurs Early During Neoplastic Tranformation and is a Target of the Growth Inhibitory Effects of TGF-β1.

Hafer LJ, AM Traish and AE Rogers, "Estrogen and Progesterone Receptors in Normal and Malignant Mammary Glands in Tea and High Fat Diet-Fed Female Sprague-Dawley Rats", 1999 Henry I Russek Student Achievement Day (Boston, MA).

D.W. Kim, M.A. Sovak, G. Zanieski, G. Nonet, P. Yaswen, M. Stampfer, J. Russo, A.E. Rogers, and G.E. Sonenshein. Russek Day, April 1999, Boston University School of Medicine, Poster Presentation: "The Early Activation of NF-κB/Rel During Neoplastic Transformation of Mammary Cells."

Trombino AF, RI Near, S Yang, LJ Hafer , PI Toselli, DW Kim, AE Rogers, GE Sonenshein and DH Sherr, "Expression of the Aryl Hydrocarbon Receptor/Transcription Factor (AhR) and AhR-Regulated *CYP1* Gene Transcripts in a Rat Model of Mammary Tumorigenesis", 1999 Henry I Russek Student Achievement Day (Boston, MA).

SEMINAR PRESENTATIONS

Hafer L.J., K.E. Murphy, A.E. Rogers and A.M. Traish, "Black Tea and Estrogen and Progesterone Receptors in the Rat Mammary Gland", 1997 Department of Defense Breast Cancer Research Program Meeting (Washington, DC), platform presentation.

Hafer, L.J., "The Effects of Dietary Fat and Black Tea on Steroid Hormone Receptors in Normal and Neoplastic Mammary Glands in Rats" BUMC, Department of Pathology and Laboratory Medicine seminar (Boston, MA), November 1997.

Hafer L.J., "Black Tea and DMBA-Induced Mammary Gland Cancer", Breast Cancer Working Group seminar of the Program in Research on Women's Health (BUMC), April 1997,.

Sonenshein, G.E., "Role of NF-κB and c-*myc* in control of Apoptosis"—Massachusetts General Hospital Cancer Center seminar, April, 1997.

Sonenshein, G.E., "Roles of NF-κB and c-Myc in Control of Cell Proliferation and Survival."—Institut Curie seminar, Paris, France, July, 1998

Sonenshein, G.E., "Roles of NF-κB and c-Myc in Control of Cell Proliferation and Survival."—Pasteur Institute seminar, Paris, France, July, 1998

Sonenshein, G.E., "Roles of NF-κB and c-Myc in Control of Cell Proliferation and Survival."—Univ. of Mass./Amherst, Dept. of Veterinarian & Animal Sciences seminar, April, 1999.

Sonenshein, G.E., "Roles of NF-κB/Rel and c-Myc in Control of Cell Proliferation."—BUMC, Arthritis Grand Rounds, February, 1999

Sonenshein, G.E., "From the Lab to the Louvre"—BUMC, Dept. of Biochemistry seminar, April, 1999.

Sonenshein, G.E., "Roles of NF-κB/Rel and c-Myc in Control of Cell Proliferation." University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, New Jersey, April 29, 1999.

DEGREES OBTAINED

Mika A. Sovak, M.D./Ph.D. degrees, 1999
Koren Mann, Ph.D. degree, 1999
Dong Kim, research for Ph.D. degree, 1999
Kristine Murphy, MA degree, 1997
Natalia Rost, MA degree, 1998

FUNDING APPLIED FOR BASED ON WORK SUPPORTED BY THIS AWARD

Awarded Grants

BC961403 (Sonenshein) 1/15/98 - 1/14/01
US Army, Breast Cancer Initiative $52,579
"Role of NF-κB/Rel in the Etiology of Breast Cancer"

1 R01 CA82742-01 7/1/99 - 6/30/04
NIH/NCI $169,527
"Role of NF-κB/Rel in the Pathogenesis of Breast Cancer"

Pending Applications

1PO1 CA 80059-01A1 (Sonenshein) 12/1/99-11/30/04
NIH/NCI
"Signaling Pathways in Stages of Mammary Tumorigenesis" $679,234 (total)
Includes projects headed by Drs. Sherr and Sonenshein and Animal Biomodel Core headed by Dr. Rogers.

RO1 (Sherr) 7/1/00-6/30/05 $160,000
NIH/NIEHS
"Role of the aromatic hydrocarbon receptor in the etiology of breast cancer"

Mass. Dept. of Public Health (Quadri) 1/1/00-12/31/02 $50,000
"Evaluation of AhR transgenic mice"

PERSONNEL

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9. CONCLUSIONS

Overall, we have confirmed our original hypothesis that carcinogen treatment can function via a novel mechanism to induce proliferation/transformation. We have shown that PAH treatment induces increased expression of both AhR and NF-κB. The induction of NF-κB can lead directly to induction of c-*myc* expression via binding to the two promoter/exon1 elements that we have localized previously. Furthermore, we have identified another entirely new mechanism whereby the p65 subunit of NF-κB acts synergistically with the AhR to induce transcription of the c-*myc* promoter. Thus, overall these studies indicate AhR and NF-κB represent major factors in control of breast cancer cell proliferation and survival. Given that NF-κB/Rel factors can be inactivated via anti-oxidants as well as by more specific inhibitory proteins, our work indicates that they represent an important new target for breast cancer therapy. Furthermore, our findings suggest the intriguing possibility that the p65 NF-κB/Rel subunit and nmt55 represent new biomarkers for the progression of breast disease.

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Black tea and mammary gland carcinogenesis by 7,12-dimethylbenz[*a*]anthracene in rats fed control or high fat diets

Adrianne E.Rogers[1], Laurie J.Hafer, Yvette S.Iskander and Shi Yang

Mallory Institute of Pathology and Department of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, MA 02118, USA

[1]To whom correspondence should be addressed
Email: aerogers@BU.edu

Epidemiological studies suggest that tea may reduce cancer risk, and in laboratory rodents, chemopreventive effects of tea or purified extracts of tea have been demonstrated in lung, gastrointestinal tract and skin. There is some evidence of chemoprevention by tea in the mammary gland, but the data are not conclusive. In order to evaluate more fully the possible influence of black tea on 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary gland tumors in the female S–D (Sprague–Dawley) rat, three large studies were performed: experiment 1, tumorigenesis in rats fed AIN-76A diet and given 25 mg/kg DMBA and 1.25 or 2.5% whole tea extract or water to drink; experiment 2, tumorigenesis in rats given 15 mg/kg DMBA and the same diet and fluids as in experiment 1; experiment 3, tumorigenesis in rats fed control or HF (high fat, corn oil) diet and given 15 mg/kg DMBA and 2% tea or water to drink. Tea was given throughout the experiment; DMBA was given by gastric gavage at 8 weeks of age. There was no consistent effect of tea on tumorigenesis in rats fed AIN-76A diet; there was, however, evidence in experiment 3 of a reduction of tumorigenesis by tea in rats fed the HF diet. In experiment 3, rats fed the HF diet and given water showed the expected increase in tumor burden (number and weight) compared with rats fed control diet. However, rats fed the HF diet and given 2% tea showed no increase in tumor burden; their tumor burden was significantly lower than in rats fed the HF diet and given water ($P < 0.01$) and was not different from rats fed control diet and given water or tea. In addition, in experiment 3, the number of malignant tumors per tumor-bearing rat was increased by the HF diet in water-drinking rats ($P < 0.01$) but not in tea-drinking rats. Therefore, it appears that tea partially blocked the promotion of DMBA-induced mammary tumorigenesis by the HF diet.

Introduction

Epidemiological studies strongly suggest that diet components are responsible in part for geographic and cultural differences in cancer site and incidence. For example, fruits and vegetables, soybeans, grains and tea are thought to contain nutrient and non-nutrient substances that reduce cancer risk in the breast, prostate and gastrointestinal tract; in contrast, fats are postulated to increase cancer risk at the same and other sites (1–5). These and other diet components have been evaluated in laboratory animal tumor models (1,5–8). The diet components considered here in relation to breast cancer, namely black tea and fats high in N-6-polyunsaturated fatty acids (N-6-PUFA*), are consumed in large amounts by people in many parts of the world and may influence cancer risk.

Recent publications of epidemiological studies indicate a possible reduction of esophageal, rectal, pancreatic and colon cancer risk associated with green tea consumption in China; studies in western populations have not yielded consistent results on effects of black tea on cancer risk at any tissue site (9–16). Epidemiological data on tea and cancer risk have been extensively reviewed recently by Yang *et al.* (9) who concluded that the data are suggestive of cancer risk reduction by tea at some sites but are not consistent. Kohlmeier *et al.* (11) concluded that there may be some protection by tea in high cancer risk groups, but the evidence is weak.

In laboratory rodents, extracts of green or black tea given in place of drinking water or added to feed reduce carcinogenesis by certain nitrosamine and polycyclic aromatic hydrocarbon carcinogens in the lung, gastrointestinal tract, liver and skin, and by UV light in the skin (9,10,17–21). Whole aqueous extracts, decaffeinated extracts and purified components of tea have been found effective to varying degrees in the different tumor models. The most extensively studied preparations reported have been aqueous extracts of green tea and its major polyphenol, (–)-epigallocatechin-3-gallate (EGCG), both of which have chemopreventive activity in the organs listed. Mechanisms postulated for the anti-carcinogenic effects of tea extracts include antioxidant activity and alteration of xenobiotic metabolism (9,10).

The epidemiological evidence for an effect of total dietary fat intake on breast cancer risk is not consistent, but a recent review (2) concluded that the weight of evidence is that postmenopausal breast cancer risk is associated with increased dietary fat intake. Attempts to detect age, endocrinological and body size characteristics as well as tumor characteristics that might clarify a relationship between dietary fat and breast cancer risk or mortality have been suggestive of increased risk of mortality with higher fat intakes (22,23) or have yielded negative results (24,25). Further studies of interactions among menopausal status, body mass and serum hormones (26,27) may contribute to clarification of the effects of dietary fat on breast cancer risk.

Diets high in N-6-PUFA are consistent and relatively powerful promoters of mammary gland tumorigenesis in laboratory rodents. Other types of fat also may be promoters. N-6-PUFA and other fats may enhance initiation of tumors as well as promotion, but their greatest and most consistent effects are as promoters (1,7,8,28).

Effects of tea have not been evaluated fully in rodent breast cancer models. The experiments that have been reported

*Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; EGCG, (–)-epigallocatechin-3-gallate; ER, estrogen receptor; HF, high fat; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MMTV, murine mammary tumor virus; N-6-PUFA, N-6-polyunsaturated fatty acids; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; S–D, Sprague–Dawley.

generally have yielded data that suggest protection by tea or its components but have not shown statistically significant effects. Hirose *et al.* (29) reported that female Sprague–Dawley (S–D) rats given 50 mg/kg 7,12-dimethylbenz[*a*]anthracene (DMBA) at 7 weeks of age and, beginning one week later, fed a natural product diet containing 1% green tea catechins (of which 53.9% was EGCG) survived longer and showed somewhat increased tumor latency and decreased tumor size compared with controls not fed tea. They reported a similar result in female F344 rats fed 2% 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (PhIP) plus 1% green tea catechins in a natural product diet (30). Weisburger *et al.* (31) reported that female S–D rats fed a high N-6-PUFA (23.5% corn oil) diet that promotes mammary tumorigenesis, given 1.25% black tea extract to drink from 6 weeks of age, and given 5 mg DMBA (equivalent to ~25 mg/kg) by gavage at 7 weeks of age, had fewer mammary fibroadenomas than rats given water; both groups had similar numbers of adenocarcinomas. In the same laboratory in the 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) mammary tumorigenesis model, tea-drinking rats developed more tumors than controls, but the tumors were smaller and had longer latencies than in controls. Consumption of 1% black tea in a purified diet for 2 weeks before DMBA exposure reduced DMBA–DNA adducts in the mammary glands of S–D rats (32), which would predict an effect of tea on initiation of carcinogenesis.

Fujiki *et al.* (33) found no effect of 0.1% EGCG in drinking water on murine mammary tumor virus (MMTV) tumorigenesis in SHN mice; Sakata *et al.* (34) found no effect of green tea extract (0.1 and 0.05%) in drinking water in the same model.

Liao *et al.* (35) reported that i.p. daily injection of EGCG (1 mg) inhibited growth of tumors from human breast cancer MCF-7 cells implanted subcutaneously in BALB/c female nude mice that carried 17-β estradiol implants. Komori *et al.* (36) reported that green tea catechins (EGCG 85%) and whole green tea extract inhibited growth of two human breast cancer cell lines (MCF-7 and BT20) in culture.

In summary, there is suggestive evidence that green tea catechins or whole green or black tea extracts reduce DMBA- or PHIP-induced mammary tumorigenesis in female rats and that similar preparations inhibit growth of human breast cancer cell lines transplanted into mice or cultured *in vitro*. Similar tea preparations did not reduce tumorigenesis by IQ in rats or by MMTV in SHN mice.

In order to evaluate more fully the possible influence of black tea on DMBA-induced mammary gland tumors in the female S–D rat, three large studies were performed: experiment 1, comparison of tumorigenesis in rats fed AIN-76A diet and given 25 mg/kg DMBA and 1.25 or 2.5% tea extract or water to drink; experiment 2, comparison of tumorigenesis in rats given 15 mg/kg DMBA and the same diet and drinking fluids as in experiment 1; and experiment 3, comparison of tumorigenesis in rats fed control or high fat (HF) diet, and given 15 mg/kg DMBA and either 2% tea or water to drink.

Materials and methods

Female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), 4–5 weeks of age, were housed individually in environmentally controlled animal quarters and handled according to the NIH guidelines. They were fed AIN-76A diet throughout the experiment (experiments 1 and 2) or fed AIN-76A diet before DMBA exposure and then, 96 h later, divided into groups and fed either AIN-76A diet, or an HF diet that was nutritionally equivalent on a caloric basis to the AIN-76A diet (experiment 3). The HF diet contained 24% vitamin-free casein, 24% corn oil, 30.8% sucrose, 9.3% corn-starch, 1.2% AIN vitamin mix, 4.2% AIN mineral mix, 0.24% choline bitartrate, 0.36% DL-methionine and 5.9% α-cellulose.

Table I. Treatment groups

Experiment	Group	No. of rats	Tea	Water	DMBA[a]
1 and 2[b]	C1	10	0	+	0
	C2	40	0	+	+
	C3	40	0	+	+
	T1	10	2.5%	0	0
	T2	40	1.25%	0	+
	T3	40	2.5%	0	+
3[c]	C1	10	0	+	0
	C4	20	0	+	+
	CF4	30	0	+	+
	T1	10	2%	0	0
	T4	20	2%	0	+
	TF4	30	2%	0	+

[a]Experiment 1, 25 mg/kg in 0.2 ml sesame oil by gastric gavage at 8 weeks of age; experiments 2 and 3, 15 mg/kg in 0.2 ml sesame oil by gastric gavage at 8 weeks of age.
[b]All rats fed AIN-76A diet throughout with free access except C3 rats; they were matched and individually pair-fed to T3 rats.
[c]All rats were fed AIN-76A diet before and until 96 h after DMBA administration; from that time until termination of the experiment, CF4 and TF4 rats were fed the high N-6-PUFA diet. All rats had free access to feed.

Black tea (World Blend Tea, Southern Tea Co., Marietta, GA) was formulated and supplied under the auspices of the Tea Trade Health Research Association. The tea was a mixture of leaves grown and processed in the major tea growing countries; the percent composition from each source was constant in all lots. A different lot was used in each of the three experiments; only one lot was used for an experiment The tea (2.5% in experiments 1 and 2, 2% in experiment 3) was brewed three times per week in a Bunn® automatic basket tea maker using deionized water and was supplied fresh in calibrated bottles to the rats at that concentration or diluted to 1.25% (experiments 1 and 2). Two days later the remaining tea was measured, and fresh tea was given. Controls were given water from the same deionizing system on the same schedule. The rats were introduced to tea in increasing concentrations over a two-week period beginning at their entry into the laboratory in experiment 1. This proved unnecessary and was not done in subsequent experiments.

The groups are summarized in Table I. In experiments 1 and 2, there were four DMBA-treated groups: two drinking water (C2 and C3) and two drinking tea (T2 and T3). Group T2 rats drank 1.25% tea, and they and C2 rats were given unlimited access to feed. Group T3 rats drank 2.5% tea and had unlimited access to feed; group C3 rats were individually matched by weight to T3 rats and pair-fed three times/week to the matched rat throughout the experiment There were, in addition, two groups, 10 rats each, not given DMBA and given water (C1) or 2.5% tea (T1) and free access to feed. In experiment 3 there were four DMBA-treated groups, two fed AIN-76A diet and given either 2.0% tea (T4) or water (C4) and two fed the HF diet and given either 2.0% tea (TF4) or water (CF4).

In all experiments, the rats were weighed weekly. DMBA (25 mg/kg in experiment 1, or 15 mg/kg in experiments 2 and 3) in 0.2 ml sesame oil, was administered by gastric gavage in a single dose to rats 8 weeks of age. Beginning 4 weeks later, rats were palpated weekly for tumor. Rats were killed and necropsied when they bore tumors that were 3–4 cm in diameter or were ulcerated; all rats remaining were killed by CO_2 inhalation and necropsied 16–18 weeks after DMBA administration.

All mammary glands and tumors were rapidly excised; tumors were weighed and sectioned; sections were fixed in 10% neutral buffered formalin (experiment 1) or 4% paraformaldehyde (experiments 2 and 3) or frozen on dry ice and held at −80°C for histochemical, biochemical and molecular studies (to be reported separately). Mammary glands were similarly fixed or frozen. Fixed tissues were processed, embedded, cut and stained with hematoxylin and eosin using routine methods.

Statistical analysis of results was performed using the programs SPSS 7.0 and SAS. The cumulative probability of bearing a palpable tumor over time was analyzed by Wilcoxon and Log-Rank tests. Chi-squared statistics were calculated to compare tumor incidences among groups. ANOVA statistics with appropriate post-hoc tests (Scheffe's and Tukey's B) were used to analyze and compare body weight, tumor number and tumor weight by treatment group and by pathology.

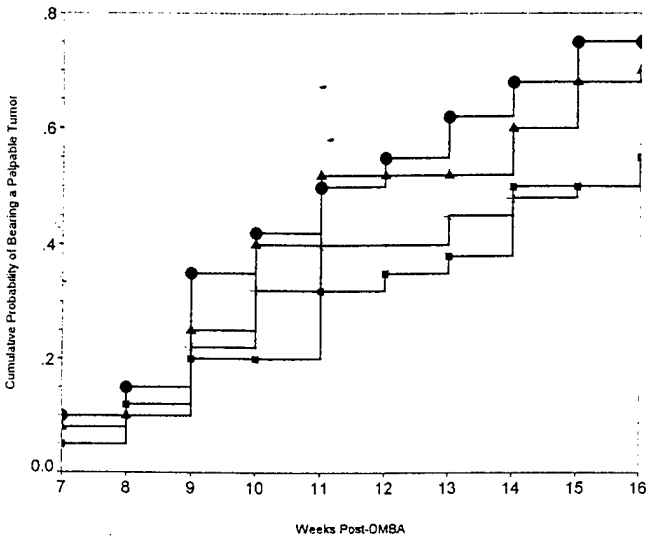

Fig. 1. Cumulative probability of bearing a mammary tumor in DMBA-treated rats in experiment 1. ■, C2; ▲, C3; ●, T2; *, T3.

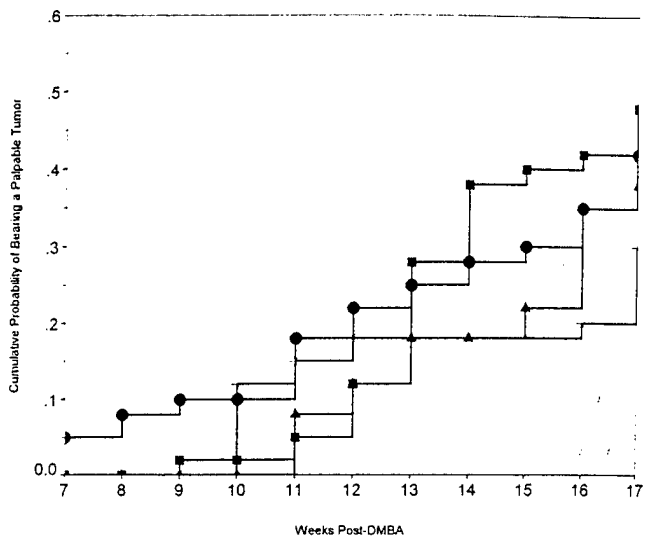

Fig. 2. Cumulative probability of bearing a mammary tumor in DMBA-treated rats in experiment 2. ■, C2; ▲, C3; ●, T2; *, T3.

Results

The rats readily accepted tea as their fluid source and ate and gained weight normally. In experiments 1 and 2, neither concentration of tea was associated with a statistical change in weight gain (data not shown). In experiment 3, rats fed the HF diet (TF4 and CF4) had identical weight gains that were slightly, but not statistically, greater than water-drinking rats fed the control AIN-76A diet (C4); tea-drinking rats fed the AIN-76A diet gained weight normally until 15 weeks of age but then showed reduced weight gain and weighed about 10% less than the C4 rats at termination of the experiment (data not shown).

Fluid intake was highly variable; in experiment 1 the tea-fed rats' daily average intake increased from 30 ml at 10 weeks of age to 38 ml at 16 weeks of age. Total tea intake over the entire experiment represented 58 ± 13 (T2) or 106 ± 22 (T3) g extracted tea leaf. The C2 and C3 groups had identical water intakes that increased on average from 41 ml at 10 weeks to 45 ml at 16 weeks of age. The fluid intakes in experiment 2 were similar in all respects to the intakes in experiment 1; intake was not measured in experiment 3.

In the three experiments, there was no consistent effect of tea on tumorigenesis in rats fed the AIN-76A diet; there was some evidence of a reduction of tumorigenesis by tea in experiment 3 in rats fed the HF diet.

Cumulative probability of tumor in experiment 1 was higher in the T2 group than in the C2 group ($P = 0.04$) and was greater, but not statistically so, in C3 than in T3 (Figure 1). In experiment 2, cumulative probabilities of tumor in both water control groups (C2 and C3) were higher than the corresponding tea-drinking groups (T2 and T3); there were no statistically significant differences. The lower dose of DMBA in experiment 2 induced a lower cumulative probability of tumor in all groups compared with experiment 1 (Figure 2).

In experiment 3, rats fed the HF diet showed the expected increase in cumulative probability of tumor compared with rats fed the control AIN-76A diet (C4 versus CF4, $P = 0.003$; T4 versus TF4, $P = 0.05$). Tea did not statistically have an effect on the cumulative probability of tumor, although TF4 was somewhat lower than CF4 (Figure 3).

In experiments 1 and 2, tumor incidence, number and weight

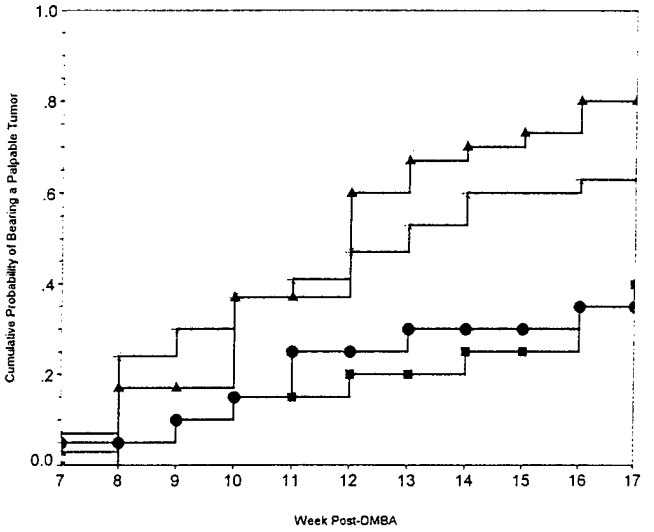

Fig. 3. Cumulative probability of bearing a mammary tumor in DMBA-treated rats in experiment 3. ■, C4; ▲, CF4; ●, T4; *, TF4.

did not differ consistently or statistically between tea-fed and water-fed rats (Table II). However, in rats fed the HF diet and given 2% tea (TF4) in experiment 3, tumor burden and total tumor weight per tumor-bearing rat, was statistically reduced ($P < 0.01$) compared with rats fed the HF diet and given water (CF4). Tumor number also was reduced in TF4 rats compared with CF4 rats, but the reduction was not significant (Table II).

In experiment 1, 85% of tumors were malignant, 62% were malignant in experiment 2, and 76% were malignant in experiment 3. In experiments 1 and 2, there was no effect of tea on the incidence of benign or malignant tumors. In experiment 3, the number of tumors and of malignant tumors per tumor-bearing rat was increased by the HF diet in water-drinking rats (C4 versus CF4, $P < 0.01$) but not in tea-drinking rats which failed to show the increase associated with the HF diet. Therefore, tea partially blocked the promotion of tumorigenesis by the HF diet.

Table II. DMBA-induced mammary tumor incidence, number and burden in female S–D rats drinking tea or water and fed control or high N-6-PUFA diets

Group	% tumor incidence[a]	No. of tumors[a,b] (per tumor-bearing rat)	Total tumor weight (g)[b] (per tumor-bearing rat)
Experiment 1[c]			
C2	60 (58)	2.9 ± 1.7 (2.4 ± 1.4)	4.0 ± 4.5
T2	75 (75)	3.4 ± 2.3 (3.0 ± 2.1)	5.6 ± 7.5
C3	62 (62)	2.8 ± 1.9 (2.2 ± 1.4)	4.7 ± 6.3
T3	68 (62)	3.2 ± 2.3 (2.7 ± 2.0)	5.4 ± 5.9
Experiment 2[c]			
C2	62 (50)	2.0 ± 1.2 (1.4 ± 1.1)	3.0 ± 3.4
T2	40 (22)	1.9 ± 1.2 (1.1 ± 1.2)	3.1 ± 4.1
C3	42 (28)	1.7 ± 0.8 (1.0 ± 1.0)	1.7 ± 3.1
T3	40 (32)	2.1 ± 1.8 (1.2 ± 1.1)	2.7 ± 3.1
Experiment 3[d]			
C4	70 (45)	1.6 ± 0.9 (1.0 ± 1.0)	1.0 ± 0.9
T4	35 (20)	2.0 ± 1.8 (1.3 ± 1.8)	1.9 ± 1.4
CF4	80 (70)	4.1 ± 2.6[e] (3.1 ± 2.2)	6.0 ± 4.1[f]
TF4	67 (50)	2.6 ± 1.5 (1.9 ± 1.6)	2.9 ± 2.8

[a]Malignant tumor incidence and number in parentheses.
[b]Mean ± standard deviation.
[c]Forty rats per group; C2, water; T2, 1.25% tea; C3, water, pair-fed to T3; T3, 2.5% tea.
[d]C4 and T4, 20 rats each; CF4 and TF4, 30 rats each; C4, water; T4, 2% tea; CF4, high fat diet + water; TF4, high fat diet + 2% tea.
[e]Significantly greater than C4 ($P < 0.01$) for all tumors and for malignant tumors.
[f]Significantly greater than C4 ($P = 0.001$) and than TF4 ($P = 0.01$).

Discussion

In the three bioassay experiments, the model responded as expected to DMBA at the two doses given and to the amount of corn oil in the diet (7,8). The ingestion of black tea had no consistent effect on mammary gland carcinogenesis in rats fed the AIN-76A diet, but tea did reduce tumorigenesis somewhat in rats fed the HF diet. In this group (TF4, experiment 3) tea ingestion significantly reduced tumor burden compared with rats drinking water. The group had also a somewhat reduced cumulative probability of bearing a tumor, but the reduction was not statistically significant. The effects of tea on DMBA tumorigenesis in rats reported previously in other laboratories have also been detected as a reduction of tumor multiplicity or size, and the Weisburger *et al.* report (31) of reduced DMBA tumor multiplicity in black-tea-drinking rats was obtained in rats fed a high corn oil diet. Therefore, it appears that black tea may reduce DMBA mammary tumorigenesis in rats, particulary if they are eating a diet high in N-6-PUFA.

Since the rats were ingesting tea before and after DMBA exposure, an effect on initiation, promotion or progression could have been detected in any of the experiments. The results of experiment 3 suggest that tea may reduce the well-known promoting effect of N-6-PUFA on mammary gland tumorigenesis (1,7,8,28). The significance of this effect has been re-emphasized by a recent updated meta analysis of data from a large number of studies in rats and mice (37). The mechanism by which N-6-PUFA promotes mammary tumorigenesis is unknown, despite many investigations of endocrine-related and other hypotheses (1,28,38). Further studies in this model might be enhanced by exploration of interactions of N-6-PUFA with tea.

Black tea intake, characterized in a large cohort of people in the Netherlands, was, at the highest level established of ⩾5 cups/day, 525 ml or ~0.03 ml/cm^2/day of 1% tea (39). The rats ingested an average of 0.1 ml/cm^2/day of 1.25 or 2.5% tea. Therefore, one can conclude that adequate and not grossly exessive levels of intake were tested.

Mammary gland carcinogenesis is highly sensitive to dietary energy supply as well as to specific fats. The concentration of tea (2.0%) used in experiment 3 had no significant effect on body weight in rats fed the HF diet (TF4) compared with controls (CF4); therefore, the reduction in tumor number in TF4 rats is not attributable to a change in weight gain.

Whole coffee extract and caffeine, an alkaloid component of tea and coffee, have been studied in the S–D rat DMBA tumorigenesis model. The results vary somewhat with timing and amount of coffee or caffeine ingestion, but they generally indicate reduction of tumor multiplicity and no change in incidence or latency when coffee or caffeine is given at initiation and no effect on promotion (40,41). Since caffeine may be present in polyphenol extracts of tea (21), it could contribute to the reported effects of tea and its extracts.

Interactions of tea or tea extracts with the estrogen receptor (ER) in the mammary glands of rats in the experiment reported here and with the ER of calf uterus *in vitro* have been found (42). Such effects have been suggested by other studies (35).

Further studies in this model, using both tea preparations and high N-6-PUFA diets, should yield information useful in chemoprevention and in understanding the basic mechanisms in mammary gland tumorigenesis.

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Expression of the Aryl Hydrocarbon Receptor/Transcription Factor (AhR) and AhR-Regulated *CYP1* Gene Transcripts in a Rat Model of Mammary Tumorigenesis[1]

Anthony F. Trombino*, Richard I. Near[+], Raymond A. Matulka[+], Shi Yang*, Laurie J. Hafer*, Paul A. Toselli[#], Dong W. Kim[#], Adrianne E. Rogers*[+], Gail E. Sonenshein[#], and David H. Sherr*[+]

[+]Department of Environmental Health, *Department of Pathology and Laboratory Medicine, [#]Department of Biochemistry, Boston University Schools of Medicine and Public Health, 80 E. Concord St., Boston MA 02118.

Running Title: AhR and CYP1 hyper-expression in rat breast tumors

Key Words: Aryl hydrocarbon receptor, breast tumors, CYP1, tumorigenesis

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[2]Address correspondence to David H. Sherr, Department of Environmental Health, Boston University Schools of Medicine and Public Health, 80 East Concord Street (S-105), Boston, MA 02118.

[3]Abbreviations: AhR: Aryl hydrocarbon receptor/transcription factor; ARNT: Aryl hydrocarbon receptor nuclear translocator protein; *CYP1*: genes encoding P450-1 proteins; DMBA: 7,12-dimethylbenz[*a*]anthracene; IHC: Immunohistochemistry; PAH: polycyclic aromatic hydrocarbon(s).

Abstract:

It has been suggested that exposure to and bio-accumulation of ubiquitous environmental chemicals, such as polycyclic aromatic hydrocarbons (PAH), contributes to human breast cancer. In animal models, PAH induce tumors in part by activating the aryl hydrocarbon receptor (AhR)/transcription factor. Historically, investigations into the role that the AhR plays in carcinogenesis have focused on its transcriptional regulation of cytochrome P450 (CYP) enzymes which oxidize PAH to mutagenic intermediates that initiate transformation. However, recent studies suggest that the AhR may directly regulate cell growth and/or function. Given the postulated role of the AhR in tumor initiation and growth, we predicted that: 1) significant levels of AhR would be expressed in tissue predisposed to PAH tumorigenesis and 2) aberrant AhR and/or AhR-regulated gene expression would accompany malignant transformation. To test these hypotheses, AhR protein and *mRNA* levels and *CYP1 m*RNA expression were evaluated in an animal model of breast cancer. Rat mammary tumors were induced by oral gavage with 15 or 25 mg/kg 7,12-dimethylbenz[*a*]anthracene (DMBA), a prototypic PAH and AhR ligand. AhR-specific immunoblotting and immunohistochemistry demonstrated modest levels of AhR protein localized to myoepithelial cells and, to a lesser extent, ductal epithelial cells in normal adult rat mammary tissue. In contrast, high levels of AhR were detected in DMBA-induced fibroadenomas, papillomas, and adenocarcinomas. AhR hyper-expression characterized both tumor cells and stromal cells. Nuclear AhR localization in tumors was suggestive of constitutive AhR activation. I*n situ* hybridization studies were consistent with high level *AhR* mRNA expression in the stroma and in neoplastic epithelial cells. Quantitative RT-PCR assays indicated a 27.6 fold increase in *AhR* mRNA in neoplastic as compared with normal rat mammary tissue. While both AhR-regulated *CYP1A1* and *CYP1B1* mRNAs were significantly induced in rat breast tissue within 6 hours of DMBA gavage, only *CYP1B1* mRNA was elevated in tumors 16-18 weeks after DMBA exposure. Collectively, these results: 1) help explain targeting of breast tissue by carcinogenic PAH, 2) imply that AhR and CYP1B1 hyper-expression represent molecular biomarkers for, at least, PAH-induced mammary cell transformation, and 3) suggest mechanisms through which the AhR and AhR-regulated gene products may contribute to carcinogenesis well after exogenous AhR ligands have been eliminated.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous environmental pollutants produced by the incomplete combustion of fossil fuels. The hypothesis that PAH, or related environmental chemicals (e.g. polychlorinated biphenyls and dioxins), are important cancer-causing agents is supported by studies demonstrating that these chemicals are powerful initiating and/or promoting agents (1-3), particularly in breast tissue (4-7). Ongoing epidemiologic studies have suggested (1, 8-16), but not proven (2, 17) a role for PAH or other lipophilic environmental chemicals in human breast cancer.

Much, if not all, of the biologic activity of PAH is mediated by the aryl hydrocarbon receptor/transcription factor (AhR)(18-20), a member of the Per-ARNT-Sim (PAS) family of transcription factors which influence development, circadian cycle, and responses to hypoxia (22-25). The AhR is complexed with heat shock protein 90 (hsp90)(20, 21) and probably other proteins (22, 23). Before and/or after ligand binding the AhR is associated with an immunophilin-like molecule (24, 25). Upon ligand binding hsp90 dissociates, the AhR translocates to the nucleus, dimerizes with a co-factor termed ARNT (aryl hydrocarbon receptor nuclear translocater), binds specific DNA transcriptional regulatory sequences (AhREs) and induces transcription of a variety of genes including those encoding growth factors and proto-oncogenes (20, 21, 26-31).

Perhaps best studied of these AhR-regulated genes are those encoding PAH-oxidizing cytochrome P450 enzymes (21, 32-35). The ability of these enzymes to promote production of DNA-reactive intermediates, such as diol epoxides, has long been recognized as one route through which AhR-regulated P450 enzymes contribute to PAH-dependent carcinogenesis (35). Furthermore, the ability of at least one P450 isoform, CYP1B1, to metabolize an endogenous substrate (17β-estradiol) into a carcinogenic metabolite (4-hydroxyestradiol) suggests an additional AhR-regulated, metabolic pathway to malignant transformation in mammary cells.

Although AhR-mediated induction of P450 enzymes has been the most frequently studied consequence of AhR activation, recent studies have begun to uncover an association between AhR activation and cell cycle. For example, elevated AhR expression is associated with mitogenic responses (36, 37), AhR activation induces *c-erb-2*, *c-myc*, *c-fos*, *c-jun* and *Ha-ras* proto-oncogenes (29, 38, 39) and CDK4 expression (40, 41), and the AhR co-immunoprecipitates with Rb, CDK4, and the p65 subunit of NF-κB (42-44). Therefore, it is possible that the AhR mediates PAH-dependent carcinogenesis both through regulation of metabolic processes and dysregulation of cell cycle.

Given this working model of AhR-dependent tumorigenesis, it would be predicted that significant levels of AhR would be expressed in tissues predisposed to PAH tumorigenesis and that aberrant AhR expression or function may accompany malignant transformation. A well-characterized model system of PAH-induced mammary tumorigenesis (5-7, 45) was employed to test these predictions. In this system, female Sprague-Dawley rats are given a single dose of a prototypic PAH, 7,12-dimethylbenz(*a*)anthracene (DMBA) by oral gavage. Fifteen to eighteen weeks later the majority of the rats develop mammary fibroadenomas, papillomas, adenomas or adenocarcinomas which closely resembled human mammary tumors (46). For the present studies, *AhR* mRNA in normal and neoplastic mammary tissue was assessed by *in situ* hybridization and quantitative RT-PCR. Similarly, AhR protein expression and cellular distribution in normal and neoplastic mammary tissue was determined by western immunoblotting and immunohistochemistry. Potential AhR activation in mammary tissue was assessed by evaluating the steady state levels of AhR-regulated *CYP1A1* and *CYP1B1* gene transcripts.

Results presented here demonstrate for the first time that normal rat mammary tissue expresses a low but significant level of AhR protein and mRNA localized primarily to mammary ductal epithelial and myoepithelial cells and that both AhR mRNA and protein are significantly elevated in DMBA-induced mammary tumors as well as in stromal elements surrounding these tumors. Furthermore, while both *CYP1A1* and *CYP1B1* transcripts are induced shortly after DMBA gavage, a preferential induction of mRNA encoding the *CYP1B1* P450 isoform is observed in mammary tumors. The contribution of

AhR expression to PAH susceptibility and the possibility that high *CYP1B1* mRNA levels in mammary tumors reflect constitutive AhR activation are addressed.

Materials and Methods

Animal treatment: Four week old female Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed individually in environmentally controlled animal quarters and maintained according to NIH Guidelines. Rats were fed *ad libitum* a nutritionally complete diet (AIN-75A) and given deionized drinking water. Rats were randomized to control and DMBA-treated groups of 5-40 animals each. At 56 days of age the experimental group was given 15 or 25 mg/kg DMBA in 0.2 ml corn oil by oral gavage, and the control group was given only the oil vehicle. Rats were observed for mammary tumors over the next 6-18 weeks. Rats were sacrificed 6 hours after vehicle or DMBA-treatment, or at the time of tumor formation (approximately 16-18 weeks) and necropsied. Mammary tissue was excised and processed as described below.

Immunohistochemistry (IHC): Portions of freshly isolated tissue were fixed in 10% formalin, embedded in paraffin, and sectioned at 4 μm for histological (hematoxylin and eosin staining) and IHC analyses. For AhR localization studies (47), slides were quenched with 3% H_2O_2 in water for 10 min at room temperature, suspended in 0.01 mM citrate buffer, and treated in a microwave oven for approximately 30 min. to retrieve antigen. Slides were then incubated with 2 μg/ml rabbit polyclonal anti-AhR antibodies (generously provided by Dr. R. Polenz) or control rabbit immunoglobulin for 1 hour at 37° C, washed and incubated with a 1:300 dilution of biotinylated swine anti-rabbit immunoglobulin antibody for 30 min. at 37° C. Slides were washed, and horse radish peroxidase-conjugated strepavidin added for a 25 min. incubation at 37° C. AhR-specific stain was visualized by treatment with DAB (DAKO, Corp., Carpinteria, CA) at room temperature for 10 min. Slides were counterstained with hematoxylin, dehydrated, and coverslipped for light microscopy. AhR staining under these conditions was completely inhibited by absorbing anti-AhR antibody with sepharose beads conjugated with recombinant AhR protein. The stain was not affected by absorbing anti-AhR antibody with sepharose beads conjugated with an irrelevant protein, lysozyme. Recombinant murine AhR used to confirm IHC specificity was produced by cloning full length murine *AhR* cDNA from *pcDNA-AhR*, provided by Dr. C. Bradfield, into the HINDIII site of *pET-25b* expression plasmid (Novagen, Inc., Madison, WI). AhR protein was then expressed in *E. Coli* BL2(DE3)pLysS with induction by 1 mM IPTG. AhR protein was purified on a nickel-agarose column (Novagen, Inc.) prior to coupling to activated sepharose 4B (Sigma Chemical Co., St. Louis, MO).

Semi-quantitative RT-PCR: Mammary tissue was frozen and pulverized into a fine powder. Total cellular RNA was isolated using RNAzol as described by the manufacturer (Leedo Medical Laboratories, Houston, TX). RNA was quantitated with a spectrophotometer at an OD of 260 and 280. Each RT-PCR reaction was performed with 5 μg RNA as described by the RT-PCR kit manufacturer (SuperScript Preamplification System; Gibco/BRL, Gaithersburg, MD). Equal sample loading was confirmed by comparison with the *β-actin* gene transcript.

The following primers and conditions were used:
AhR: 5'-CTGGCAATGAATTTCCAAGGGAGG and 5'-CTTTCTCCAGTCTTAATCATGCG in 1.5 mM $MgCl_2$ for 35 cycles to yield a 334 base pair product; *CYP1A1*:5'-TCTGGAGACCTTCCGGCATT and 5'-CCGTTCGCTTTCACATGCCG in 1.5 mM $MgCl_2$ for 36 cycles to yield a 260 base pair product; *CYP1B1*: 5'-TCAACCGCAACTTCAGCAACTTC and 5'AGGTGTTGGCAGTGGTGGCAT in 1.5 mM $MgCl_2$ for 36 cycles to yield a 404 base pair product; *β-actin*: 5'GTCGTCGACAACGGCTCCGGCATGTG and 5'-CATTGTAGAAGGTGTGGTGCCAGATC, 1mM $MgCl_2$ for 30 cycles to yield a 256 base pair product. Amplified cDNAs were electrophoresed through 3% agarose gels (3:1 NuSieve:Le agarose, FMC, Rockland, ME) and visualized by ethidium bromide staining. The number of PCR cycles was adjusted such that resulting band densities fell on the linear portion of the logarithmic amplification curve. *AhR*, *CYP1A1*, and *CYP1B1* cDNA band densities

were determined by image analysis with Kodak Digital Science 1D imaging software and presented as the average ratio of experimental band density/β-actin band density ± standard error.

Quantitative competitive RT-PCR: An irrelevant 400 base pair bacterial DNA fragment was combined with hybrid primers having complementary sequences to the AhR primer oligonucleotides, cloned, and used as a competitive inhibitor ("mimic") of the *AhR* cDNA during PCR (48). Known quantities of mimic (0.05-60 fg/μl) were titered into PCR mixtures containing *AhR* cDNA. cDNA mixtures were then amplified, products electrophoresed, and visualized with ethidium bromide. Band densities were determined with imaging software and plotted as a function of the concentration of competitive inhibitor. The interpolated cross-over point where AhR and mimic band densities were equal indicated the concentration of *AhR* cDNA and, assuming efficient reverse transcription, *AhR* mRNA.

Western immunoblotting: Mammary tissue was suspended in lysing buffer (1% Triton X-100, 150 mM NaCl, 25 mM Tris/HCl, 1 μg/ml aprotenin, 10 μg/ml leupeptin, 1 mM EDTA, 50 mM NaF, 1 mM orthovanadate, 1 mM PMSF) and homogenized with a Dounce homogenizer. Cell lysates were centrifuged for 15 seconds at 15,000 x G and supernatants containing cytosolic protein frozen at -20° C.

Supernatant protein concentrations were first measured with a bicinchoninic acid protein assay reagent kit (Pierce Chemical Co., Rockford, IL). Whole protein (40 μg) samples were diluted in Laemmli buffer and loaded into 10% SDS polyacrylamide gels. Electrophoresis was carried out at 150 volts for 1 hour. Proteins were transferred to nitrocellulose filters (Bio-Rad Hercules, CA) at 150 mA for 1 hour or at 30 mA overnight. Transfer was monitored by staining proteins with 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid solution (Sigma Chemical Co., St. Louis, MO). Ponceau S was washed out with double distilled water and TBST buffer (20 mM Tris, 0.5 M NaCl, 0.03% Tween 20, pH 7.5). Filters were blocked with TBST buffer containing 5% (w/v) nonfat dry milk, washed twice for 5 min. in TBST, and incubated with monoclonal anti-AhR antibody Rpt-9 (49) at a 1:10,000 dilution for 1 hour at room temperature. Filters were washed with TBST, incubated for 1 hour at room temperature with a 1:6,000 dilution of HRP-goat anti-mouse antibody (Sigma Chemical Co.) and washed twice again. AhR protein bands were visualized by chemiluminescence (Du Pont NEN Research Products Co., Boston, MA) and band densities quantitated with Kodak DS1D imaging software. Band densities were compared using the two tailed Student's t test. Comparable results were obtained with three AhR-specific antibody preparations, two monoclonal mouse antibodies (RPT-9 and RPT-1) (49) and polyclonal rabbit antibodies from BioMole, Inc. (Plymouth Meeting, PA).

***In situ* hybridization:** *In situ* hybridization for *AhR* mRNA was performed as previously described (47). Mammary tissue was fixed with 4% paraformaldehyde, paraffin embedded, sectioned, dehydrated, and hybridized for 18 hr. at 52° C with either sense or anti-sense riboprobes. To generate sense and anti-sense probes, a 334 nt fragment of rat *AhR* cDNA was cloned into *pGEM-T* (Promega, Madison, WI). RT-PCR was performed with rat RNA using the primers 5'-CTGGCAATGAATTTCCAAGGGAGG-3' and 5'-CTTTCTCCAGTCTTAATCATGCG-3' and the Superscript Preamplifcation Kit and Taq polymerase.(Gibco/BRL Products) as described by the manufacturer. The amplified fragment was subcloned into *pGEM-T* by the A-T tailing method as described by the manufacturer (Promega, Inc.). ^{35}S radiolabelled *AhR* riboprobes were generated using *T7* (sense) and *SP6* (anti-sense) promoters with linearized *Xba I* and *HindIII* digests of the rat cDNA template. Slides were washed and auto-radiographs exposed for 6 weeks.

Results

AhR expression in normal and neoplastic rat mammary tissue:

The ability to induce a number of tumor types with AhR ligands in animal models and the recent association between AhR activation, intracellular signaling, and control of cell cycle (29, 36-44) suggest that tissues targeted by and tumors induced with PAH would express significant AhR levels. To test this

hypothesis, a well-characterized rat model of DMBA-induced mammary tumors was exploited (6, 7). For each of three experiments, eight week old female Sprague-Dawley rats were randomized into two groups. Experimental groups were given either 25 mg/kg (experiment 1) or 15 mg/kg DMBA (experiments 2 and 3) in 0.2 ml corn oil by oral gavage. Control groups received vehicle alone. Tumors were detected in 60% - 70% of the DMBA-treated rats within 16-18 weeks. Rats were sacrificed and necropsied at that time. Results described below were similar in all 3 experiments.

To determine the cellular distribution of AhR in mammary tissue, normal and neoplastic mammary tissues from vehicle and DMBA-treated rats respectively were sectioned and treated with polyclonal AhR-specific antibody, AhR-specific antibody absorbed with lysozyme-coupled sepharose beads, AhR-specific antibody absorbed with recombinant AhR-coupled sepharose beads or control rabbit immunoglobulin. AhR staining was visualized with biotin-labeled anti-rabbit antibody and peroxidase conjugated strepavidin.

Significant staining was observed in normal mammary gland sections treated with AhR-specific antibody (Figure 1A). AhR protein was detected in myoepithelial cells (arrows) and, to a lesser extent, in ductal epithelial cells (arrowheads). The AhR was localized to the cytoplasm in both cell types. AhR was not detected in fibroblastic stromal cells or adipocytes. In sharp contrast, high level AhR expression was observed in mammary tumors (Figure 1B, 1C, 1D). Interestingly, significant AhR staining was detected in both tumor cells and stromal cells surrounding and within tumors (Figure 1B, 1C, 1D). Localization of this stain to tumor cell nuclei (Figure 1B, 1C, 1D, arrowheads) and fibroblastic cell nuclei (Figure 1B, 1C, 1D, arrows) suggested constitutive AhR activation in both tumor cells and stromal elements. This pattern of high level AhR expression localizing to cell nuclei in at least a subset of tumor cells and stromal cells was observed in benign fibroadenomas and papillomas, and malignant invasive and non-invasive papillary and cribriform adenocarcinomas. Pre-absorption of AhR-specific antibody with hen egg lysozyme-coupled sepharose beads had no effect on AhR staining (Figure 1D). In contrast, pre-absorption of AhR-specific antibody with recombinant AhR-coupled sepharose beads completely ablated staining (Figure 1E) . No stain was observed when normal rabbit immunoglobulin was used in place of rabbit anti-AhR antibody (Figure 1F).

To quantitate the increase in AhR expression in tumors, proteins extracted from normal and neoplastic mammary tissue were analyzed by AhR-specific immunoblotting. Relatively low level AhR expression was noted in normal mammary tissue samples from vehicle-treated rats (Figure 2, lanes 2-4), with one exception (sample 3-4, lane 1). Significantly increased levels of AhR protein were observed in DMBA-induced tumors (Figure 2, and data not shown). Analysis of AhR band densities from the 6 control and 6 neoplastic samples evaluated by western immunoblotting indicated a statistically significant 5.8 fold increase in AhR protein ($p<0.001$).

AhR mRNA expression in normal and neoplastic rat mammary tissue:

To determine if the observed increase in AhR protein in rat mammary tumors correlates with increased steady state *AhR* mRNA levels, sections of normal and neoplastic rat mammary tissue were probed by *in situ* hybridization with an *AhR* mRNA-specific ^{35}S-AhR antisense riboprobe or with a control ^{35}S-*AhR* sense riboprobe. A modest signal was observed in normal mammary tissue treated with the *AhR* antisense riboprobe (Figure 3A, 3B). Consistent with IHC studies, this signal localized exclusively to ductal epithelial and myoepithelial cells. In contrast, a notably higher signal was consistently observed in neoplastic tissue (Figure 3C, 3D). The *AhR* mRNA signal was associated with both neoplastic epithelial cells and stromal cells encircling mammary tumors. No appreciable background staining was observed with *AhR* sense riboprobe (Figure 3E and 3F).

To confirm and extend these results, RNA obtained from normal and neoplastic mammary tissue was reverse transcribed and amplified by PCR with *AhR* or, as a control, *β-actin* cDNA-specific primers. For comparison, RNA was also extracted from the murine Hepa-1c1c7 hepatoma, reverse transcribed and PCR amplified. Six randomly selected normal samples from vehicle-treated rats and twenty

randomly selected tumor samples from DMBA-treated rats were evaluated. As expected from numerous previous reports (51, 52), high levels of *AhR* mRNA were detected in Hepa-1c1c7 cells (Figure 4, lane 2). Relatively low levels of *AhR* mRNA were detected in most control mammary tissue samples (lanes 3-7), although the levels in control sample 3-4 (lane 5) approached those seen in Hepa-1c1c7. Note that control sample 3-4 also expressed relatively high levels of AhR protein (Figure 2). In contrast, the vast majority of DMBA-induced tumors expressed elevated *AhR* mRNA levels (lanes 8-14). High levels of AhR mRNA were observed in 19 of 20 tumor samples examined with no obvious differences between tumor classifications. The identification of one invasive cribriform adenocarcinoma, in which *AhR* mRNA was not detected (lane 13), suggested that lack of high level *AhR* mRNA expression in PAH-induced tumors was the exception rather than the rule. Interestingly, high level *AhR* mRNA was noted in one spontaneous noninvasive papillary adenocarcinoma arising in a 6 month old rat (lane 15/tumor 2-15-L3). The level of AhR protein in this sample was approximately 4 fold higher than levels observed in mammary tissue from control rats (not shown).

An *AhR* cDNA-specific competitive mimic, developed previously in our laboratory (47), was then used to quantitate the increase in *AhR* mRNA in mammary tumors. The results of a representative experiment comparing *AhR* mRNA from a control mammary tissue sample (3-1) with *AhR* mRNA from tumor tissue (sample 144-R5, a DMBA-induced papilloma) are presented in Figure 5. The steady-state level of *AhR* mRNA increased from 0.076 ± 0.038 fg/μg total RNA in 6 randomly selected control mammary tissue samples to 2.1 ± 0.78 fg/μg total RNA in 6 randomly selected tumor samples, a statistically significant 27.6 fold increase ($p<0.01$). In general, *AhR* mRNA levels paralleled protein expression (Figure 2). A more profound increase in steady-state mRNA levels relative to the increase in steady-state protein levels (Figure 2) in tumors tissue is consistent with the previously documented rapid turnover of activated AhR (53).

CYP1A1 and 1B1 mRNA expression in histologically normal mammary tissue 6 hours after DMBA exposure and in neoplastic mammary tissue:

Ligand-activated AhR induces transcription of cytochrome P450 enzymes in many tissues including breast (21, 54-62). Furthermore, there is a direct correlation between AhR levels and constitutive CYP1A1 and CYP1B1 expression in murine cells (63, 64). From these findings it was predicted that *CYP1* mRNA transcripts would be induced shortly after DMBA exposure and that they could remain elevated in neoplastic tissue expressing high levels of potentially active AhR. Evaluation of *CYP1A1 and CYP1B1* induction in particular is important given their involvement in the biotransformation of exogenous (e.g. DMBA) and endogenous (e.g. 17β-estradiol) substrates. Rats were treated by oral gavage with vehicle or DMBA and sacrificed 6 hours later or at the time of tumor formation. Mammary tissue RNA was extracted and *AhR*, *CYP1A1*, *CYP1B1* and, as a control, *β-actin* transcripts were assayed by semi-quantitative RT-PCR. *AhR*, *CYP1A1*, *CYP1B1* mRNA levels in Hepa-1c1c7 hepatoma cells, in a human breast cancer cell line (MCF-7) and/or in a rat mammary tumor sample (109-L2) were evaluated as positive controls.

Analyses of steady-state *AhR* mRNA levels 6 hours after DMBA gavage in histologically normal mammary gland samples indicated a small, statistically insignificant decrease in *AhR* expression relative to levels in control samples from rats given vehicle alone (Figure 6, *β-actin* normalized *AhR* band densities: Controls: 0.36 ± 0.04; DMBA-treated: 0.23 ± 0.07, NS). These results are consistent with previous reports of a decrease in AhR expression shortly after its activation *(65)*. As predicted, both *CYP1A1* and *CYP1B1* mRNA levels were significantly increased 6 hours after DMBA gavage (Figure 6, *β-actin* normalized band densities: *CYP1A1*: Control: 0 ± 0; DMBA-treated: 0.30 ± 0.09, $p<0.001$; *CYP1B1*: Control: 0.06 ± 0.01; DMBA-treated: 1.11 ± 0.15, $p<0.001$). These results strongly suggest AhR activation relatively soon after DMBA exposure. Expression of high levels of *CYP1B1* but not *CYP1A1* mRNA in rat mammary tumor sample 109-L2 is notable as is the reciprocal expression of *CYP1A1* but not *CYP1B1* mRNA in Hepa-1 cells.

As previously shown (Figures 3 and 4), steady-state *AhR* mRNA levels in neoplastic tissue samples were consistently elevated relative to levels in normal tissue from vehicle-treated controls (Figure 7). Low levels of *CYP1B1* mRNA were detected in normal mammary gland samples from vehicle-treated rats. Significantly, *CYP1B1* mRNA was detected in all 7 tumor samples, with *CYP1B1* mRNA levels in 5 of those samples being notably high (*β-actin* normalized band densities: Control: 0.07 $\pm$ 0.07; tumors: 0.28 $\pm$ 0.06, $p<0.04$). Interestingly, *CYP1B1* mRNA expression in grossly normal mammary glands from a tumor-bearing rat also appeared elevated (*β-actin* normalized *CYP1B1* band densities: DMBA-treated samples 2-39 and 2-56: 0.26 $\pm$ 0.14). In contrast, while a significant level of *CYP1A1* mRNA was observed in one normal sample (sample 3-1), *CYP1A1* mRNA was not detected in DMBA-induced tumors (lanes 8-14). These results: 1) are consistent with reciprocal chronic expression of CYP1A1 and CYP1B1 observed in different cell types (66-69), 2) indicate the predominance of the CYP1B1, DMBA-metabolizing P450 isoform in DMBA-induced mammary tumors, and 3) demonstrate a strong correlation between high level AhR expression and AhR-regulated *CYP1B1* expression during and after malignant transformation.

Discussion

Studies presented herein were designed in part: 1) to test the prediction that rat mammary tissue, a primary target of aryl hydrocarbon-induced tumorigenesis, expresses AhR, 2) to determine the cellular distribution of the AhR in mammary tissue, 3) to assess the possibility that neoplastic transformation is associated with elevated AhR expression, and 4) to determine if AhR expression in DMBA-induced mammary tumors correlates with expression of AhR-regulated *CYP1* mRNAs. The results are consistent with the hypothesis that the AhR plays a role in directing the carcinogenic effects of environmental PAH to breast tissue and suggest that both the AhR and CYP1B1 may play important ongoing roles during mammary tumorigenesis.

The first series of experiments addressed the putative role of the AhR early in the carcinogenic process. Experiments utilizing AhR-specific IHC confirmed the hypothesis that normal rat mammary tissue expresses AhR. Both myoepithelial cells and, to a lesser extent, ductal epithelial cells were shown to express cytoplasmic AhR. Mammary fibroblasts and adipocytes did not express significant AhR levels. These results imply that exposure to PAH initially contributes to tumor initiation either through direct interaction with AhR^+ epithelial cells or indirectly through interaction with adjacent AhR^+ myoepithelial cells. In the classic view of PAH-induced tumorigenesis, it would be assumed that this early event in mammary tumorigenesis is mediated by local production of mutagenic metabolites catalyzed by AhR-regulated, cytochrome P450(s) (59, 70, 71). Consistent with this model of tumor initiation, *CYP1A1* and *CYP1B1* mRNA levels in mammary tissue increased within six hours of DMBA exposure (Figure 6). Given the cellular distribution of the AhR in mammary tissue, it may then be postulated that the production of reactive metabolites by ductal epithelial cells (a direct PAH effect) or by adjacent myoepithelial cells from which metabolites diffuse (an indirect PAH effect) initiates target epithelial cells. In addition, these reactive intermediates could stimulate oxidative stress responses which acutely influence cell survival or growth. The demonstration that DMBA-induced rat mammary tumors also express high levels of constitutively active NF-κB (72), a cell survival factor induced by oxidative stress (73), is consistent with this hypothesis.

A number of additional mechanisms could contribute to the early PAH-dependent events in mammary cell transformation. Given the growing evidence supporting a role for the AhR in growth regulation, including the demonstrations that *AhR* anti-sense oligonucleotides slow cell growth and that AhR co-immunoprecipitates with Rb protein, an NF-κB subunit shown to regulate c-myc expression, and a cyclin-dependent kinase (40, 42-44, 73, 81), it could reasonably be postulated that DMBA-dependent AhR activation directly alters growth characteristics of mammary epithelial cells. Alternatively, or in addition, it is possible that AhR signaling in myoepithelial cells alters the mammary duct and terminal end bud microenvironment which in turn modifies epithelial cell growth shortly after

DMBA exposure. Indeed, extracellular matrix and growth factors elaborated by the microenvironment surrounding and supporting mammary epithelial cells have been implicated in the regulation of epithelial cell growth and in malignant transformation of epithelial cells (74-76).

Similarly, if the AhR plays a role in regulating epithelial cell growth in mammary tumors it would be predicted that tumors and/or cells of the tumor microenvironment would express significant, perhaps elevated levels of AhR protein. Western immunoblotting clearly demonstrated a profound increase in AhR expression in DMBA-induced tumors. Notably high levels of *AhR* mRNA in mammary tumors, as assessed by quantitative RT-PCR, suggested that elevated AhR expression is mediated, at least in part, by increased *AhR* mRNA transcription and/or stability. AhR-specific immunohistochemical and *in situ* hybridization analyses of tumor sections confirmed this dramatic increase in both AhR protein and mRNA. These results may be contrasted with those demonstrating that treatment of Sprague-Dawley rats with TCDD, a high affinity AhR ligand which is not well metabolized, results in long-term down-regulation of AhR in liver, lung, thymus and spleen (77).

AhR expression in tumors was localized to both tumor and surrounding stroma. The absence of detectable AhR in normal mammary tissue stroma and high AhR expression in fibroblast populations surrounding tumors is consistent with a generalized up-regulation of fibroblast AhR during tumorigenesis or in response to tumor formation rather than selection of AhR^{high} fibroblast populations. In contrast, the expression of AhR in normal mammary epithelial cells and the presumably clonal nature of the AhR^{high} cell tumors suggest that high level AhR expression in tumors could result either from selection of AhR^{high} epithelial cells, perhaps as a consequence of attendant high levels of P450 activity, or from induction of AhR expression during transformation. In addition to a potential role in growth regulation, a relatively early up-regulation of the AhR would be predicted to potentiate the effects of secondary PAH or endogenous AhR ligand exposures on epithelial cell transformation. Studies designed to test these hypotheses are underway. Regardless of the mechanisms responsible for elevated AhR expression in mammary tumors and in the tumor microenvironment, results presented here suggest that high AhR expression may be a molecular biomarker for the transformation process.

Significantly, AhR nuclear localization was observed both in mammary tumors and in the surrounding stromal populations suggesting constitutive AhR activation. This observation is particularly intriguing since the initiating ligand, DMBA, and its metabolites are either excreted or covalently adducted onto macromolecules several weeks before tumors are detected (78). Nuclear localization of the AhR in fibroblasts of the tumor microenvironment may alter fibroblast growth and/or function. Changes in fibroblast function would be significant in this context since fibroblasts produce multiple epithelial cell-directed growth factors (75, 76). The putative activation of AhR in mammary tumor cells is reminiscent of results obtained with immortalized mouse hepatoma, monkey kidney, and human epithelial carcinoma cell lines in which constitutive nuclear AhR expression (79, 80), AhR transcriptional activity (79, 81), or binding to the nuclear Rb protein (42) was observed in the absence of exogenous AhR agonists. Whether this apparent AhR activation in tumor cells is mediated by an as yet undefined endogenous AhR ligand or by factors capable of activating the AhR in the absence of a nominal ligand, this result suggests a possible role for continued AhR signaling in maintenance of tumor cell growth. This hypothesis is supported by the inhibition of human breast cancer cell line growth following AhR down-regulation (82). In this context, the documented inhibition of human or rat breast cancer cell growth with AhR antagonists or agonists (83-85) could reflect either direct inhibition of AhR-dependent growth signals or diversion of the constitutively active AhR signaling pathway, presumably towards an anti-estrogenic signaling pathway (86-88).Thus, both AhR agonists and antagonists may prove useful as chemotherapeutics.

Constitutive expression of murine CYP1A1 and CYP1B1 has been linked to the level of AhR expression (63, 64). Therefore, it was predicted that high level AhR expression and/or constitutive activation in neoplastic mammary tissue would correlate with high levels of *CYP1A1* and/or *CYP1B1* mRNA. Indeed, high constitutive expression of *CYP1B1* mRNA in tumors was noted. The

predominance of *CYP1B1* mRNA is consistent with reciprocal expression of CYP1A1 and CYP1B1 in several tissues (66) and with the predominance of CYP1B1 in several human cancers including breast carcinomas (67, 89). Therefore, as previously suggested (69), high level CYP1B1 expression, like hyper-expression of the AhR, may represent a molecular marker for carcinogenesis. Additional experiments are required to confirm that this constitutive *CYP1B1* expression is AhR-regulated and is present throughout tumorigenesis.

Finally, CYP1B1 is extremely active in the conversion of several PAH to mutagenic intermediates (90). It is also a potent 17β-estradiolhydroxylase and is capable of catalyzing the production of 4-hydroxyestradiol, a known carcinogen in multiple tissues, including mammary tissue (91, 92). Therefore, it is possible that CYP1B1 contributes to mammary carcinogenesis by bioactivation of both exogenous pro-carcinogens and endogenous estrogens. Its expression in neoplastic tissue may also have implications for bioactivation of anti-tumor chemotherapeutics.

Figure Legends

Figure 1: AhR distribution in normal rat mammary tissue and DMBA-induced mammary tumors as assessed by immunohistochemistry:

Mammary tissues from female Sprague-Dawley rats treated by oral gavage with vehicle or DMBA were fixed, embedded, sectioned and stained with control or AhR-specific antibody as described in Materials and Methods. **Panel A**: A mammary gland section from a rat treated 16-18 weeks earlier with vehicle and stained with AhR-specific antibody. Arrows denote myoepithelial cells. Arrowheads denote ductal epithelial cells. **Panel B**: A DMBA-induced invasive cribriform adenocarcinoma (68-R4) section stained with AhR-specific antibody. Arrows and arrow heads indicate nuclear staining in fibroblasts and tumor cells, respectively. **Panel C**: A DMBA-induced invasive papillary adenocarcinoma (25-L2) section stained with AhR-specific antibody. Arrows and arrow heads indicate nuclear staining in fibroblasts and tumor cells, respectively. **Panel D**: A DMBA-induced invasive cribriform adenocarcinoma with focal comedo carcinoma (106-R3) section stained with AhR-specific antibody which was pre-absorbed with lysozyme-sepharose beads. Arrows and arrow heads indicate nuclear staining in fibroblasts and tumor cells, respectively. **Panel E**: A DMBA-induced invasive cribriform adenocarcinoma (106-R3) section stained with AhR-specific antibody pre-absorbed with recombinant AhR-sepharose beads. Panel F. A DMBA-induced invasive cribriform adenocarcinoma (106-R3) section treated with normal rabbit immunoglobulin. Representative sections from a total of 10 normal mammary glands (from vehicle-treated rats) and 107 tumors (from DMBA-treated rats) are presented.

Figure 2: AhR expression in normal rat mammary tissue and DMBA-induced mammary tumors as assessed by western immunoblotting:

Protein (40 μg) extracted from normal mammary tissue (lanes 1-4) or DMBA-induced mammary tumors (lanes 5-8) were electrophoresed, transferred to nitrocellulose filters, and probed with AhR-specific antibody RPT-9. Results from the following tumor types are presented: 3-29-L3: invasive papillary and cribriform adenocarcinoma, 2-32-L1: noninvasive secretory adenocarcinoma, 2-56-L1: noninvasive papillary and cribriform adenocarcinoma, 2-47-R3: noninvasive papillary adenocarcinoma. Relative band densities in 6 randomly selected normal and 6 mammary tumor samples tested were 3348 $\pm$ 962 and 19,316 $\pm$ 2386, respectively ($p<0.001$). Data from a representative experiment are presented.

Figure 3: *AhR*-specific *in situ* hybridization of normal rat mammary tissue and DMBA-induced mammary tumors:

^{35}S-*AhR* riboprobes were generated using *T7* (sense) and *SP6* (anti-sense) primers with linearized *Xba I* and *HindIII* digests of a rat *AhR* cDNA fragment cloned in *pGEM-T* as template. Normal mammary tissue from vehicle-treated rats and mammary tumors from DMBA-treated rats were fixed, dehydrated, and hybridized for 18 hrs. at 52° C with either anti-sense (A-D) or sense (E,F) riboprobes. Slides were washed and autoradiographs exposed for 6 weeks. Tissue sections from normal mammary tissue are presented in panels A and B; sections of a DMBA-induced mammary noninvasive papillary adenocarcinoma (2-74-L2) are presented in panels C-F. Left panels: light field; Right panels: dark field.

Figure 4: *AhR* mRNA expression in normal rat mammary tissue and mammary tumors as assessed by semi-quantitative RT-PCR:

RNA (5 μg) extracted from normal mammary tissue (i.e. from vehicle-treated rats) or from mammary tumors was reverse transcribed. *AhR* and *β-actin* cDNAs were then amplified by PCR, electrophoresed through 3% gels and visualized by ethidium bromide staining. Results from the following tumor types are presented: 109-L2: papilloma with focal carcinoma; 124-R2: noninvasive cribriform and papillary adenocarcinoma; 144-R4: papilloma; 2-49-L3: invasive papillary and

cribriform adenocarcinoma; 2-56-R1: noninvasive papillary and cribriform adenocarcinoma; 124-R6: noninvasive cribriform and papillary adenocarcinoma; 3-28-R4/5: invasive papillary and cribriform adenocarcinoma; 2-15-L3: papillary and cribriform adenocarcinoma. The tumor in sample 2-15-L3 arose spontaneously in a 6 month old control rat. MSP: size markers.

Figure 5: *AhR* mRNA quantitation by competitive mimic RT-PCR:

RNA was extracted from rat normal mammary tissue (sample 3-1) or from a DMBA-induced papilloma (144-R5) and cDNA reverse transcribed. cDNA was mixed with known concentrations of an *AhR* competitive mimic cDNA (0.05-60 fg/μl) as indicated and amplified with *AhR*-specific primers. Band densities were determined and plotted as a function of the concentration of competitive inhibitor. The known concentration of mimic at the interpolated cross-over point where AhR and mimic band densities were equal was used to calculate the concentration of *AhR* cDNA and mRNA. MSP: size markers.

Figure 6: *CYP1A1* and *CYP1B1* mRNA induction in mammary tissue within six hours of DMBA gavage:

Female rats were treated by oral gavage with vehicle (corn oil) or 15 mg/kg DMBA and sacrificed 6 hours later. RNA (5 μg) extracted from mammary tissue was reverse transcribed and PCR amplified with *AhR*-, *CYP1A1*-, *CYP1B1*, and *β-actin*-specific primers. RNAs from Hepa1c1c7 (Hepa-1) and tumor sample 109-L2 (a DMBA-induced papilloma with focal carcinoma) were extracted and RT-PCR amplified in parallel as positive controls. Amplified cDNAs were electrophoresed through 3% gels and visualized by ethidium bromide staining. *AhR*-, *CYP1A1*-, *CYP1B1* band densities were normalized to *β-actin*: *AhR*: Control: 0.36 $\pm$ 0.04; DMBA-treated: 0.23 $\pm$ 0.07, NS; *CYP1A1:* Control: 0 $\pm$ 0; DMBA-treated: 0.30 $\pm$ 0.09, $p<0.001$; *CYP1B1:* Control: 0.06 $\pm$ 0.01; DMBA-treated: 1.11 $\pm$ 0.15, $p<0.001$.

Figure 7: *CYP1A1* and *CYP1B1* mRNA expression in normal rat mammary tissue and DMBA-induced mammary tumors:

Female rats were treated by oral gavage with vehicle (corn oil) 15 mg/kg or 25 mg/kg DMBA and sacrificed approximately 16 weeks later at the time tumors were palpable. RNA (5 μg) extracted from normal mammary tissue and from mammary tumors was reverse transcribed and cDNA PCR amplified with *AhR*-, *CYP1A1*-, *CYP1B1*, and *β-actin*-specific primers. RNAs from murine Hepa-1c1c7 (Hepa-1) and human MCF-7 cells were extracted, reversed transcribed and cDNA PCR amplified in parallel as positive controls. Grossly normal samples 2-39 and 2-56 were from DMBA-induced tumor-bearing rats. Amplified cDNAs were electrophoresed through 3% gels and visualized by ethidium bromide staining. *β-actin* normalized band densities: *AhR*: Control: 0.16 $\pm$ 0.02; tumors: 0.64 $\pm$ 0.06, $p<0.001$; samples 2-39 and 2-56: 0.26 $\pm$ 0.14; *CYP1B1*: Control: 0.07 $\pm$ 0.07; tumors: 0.28 $\pm$ 0.06, $p<0.04$). *CYP1B1* mRNA expression in at least 1 grossly normal mammary gland from DMBA-treated rats also appeared elevated compared to *CYP1B1* mRNA expression in control samples from vehicle-treated rats (*β-actin* normalized *CYP1B1* band densities, DMBA-treated sample 2-39: 0.40; DMBA-treated sample 2-56: 0.12). Results from the following tumor types are presented: 109-L2: papilloma with focal carcinoma; 144-R4: papilloma; 2-39-L3: noninvasive papillary and cribriform adenocarcinoma; 2-56-L1: noninvasive papillary and cribriform adenocarcinoma; 2-49-L3: invasive papillary and cribriform adenocarcinoma; 3-28-R4/5: invasive papillary and cribriform adenocarcinoma; 3-29-L3: noninvasive papillary and cribriform adenocarcinoma.

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Figure 1: AhR Distribution in Normal Rat Mammary Tissue and DMBA-Induced Mammary Tumors as Assessed by Immunohistochemistry

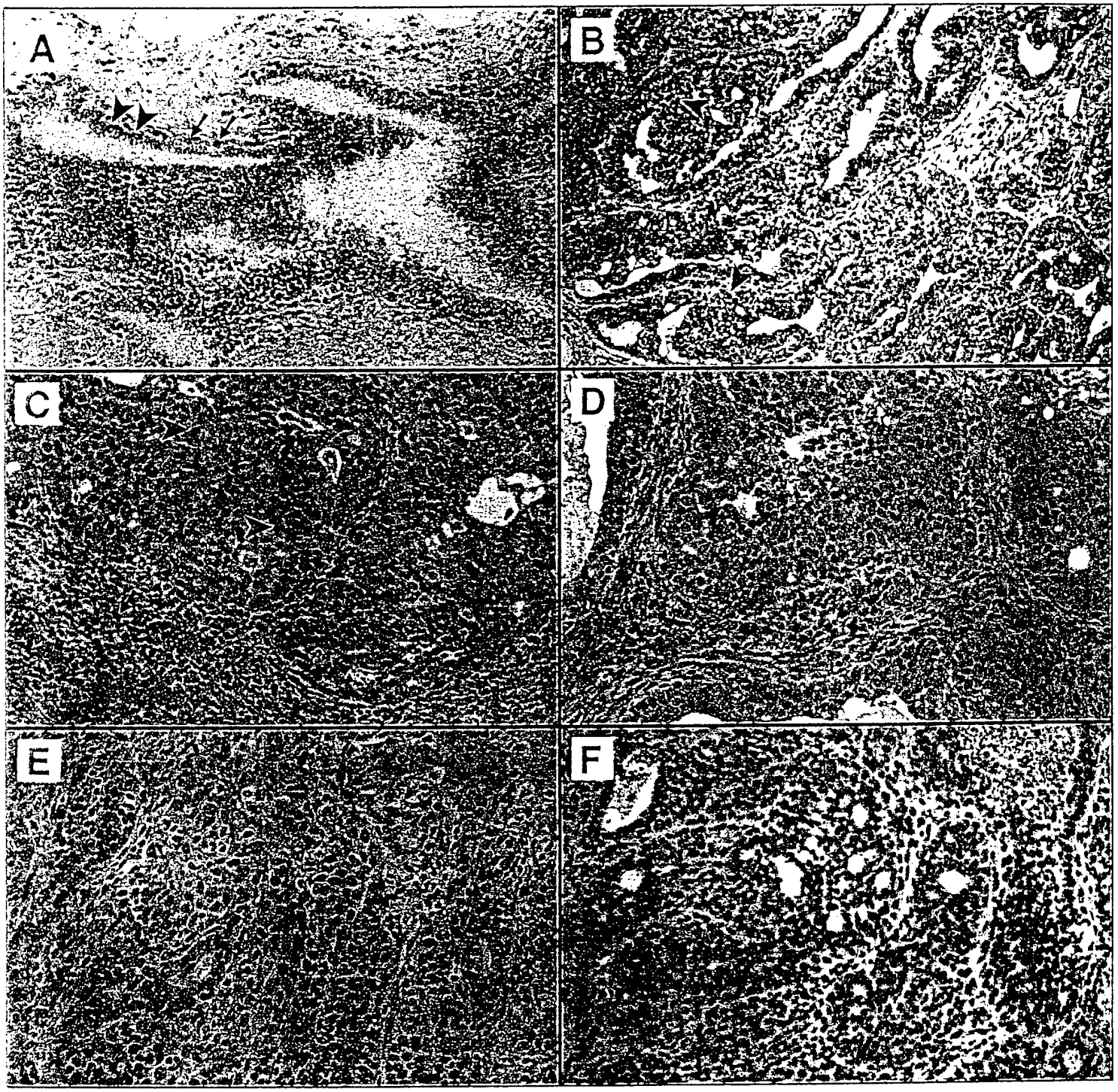


Figure 2: AhR Expression in Normal Rat Mammary Tissue and DMBA-Induced Mammary Tumors as Assessed by Western Immunoblotting

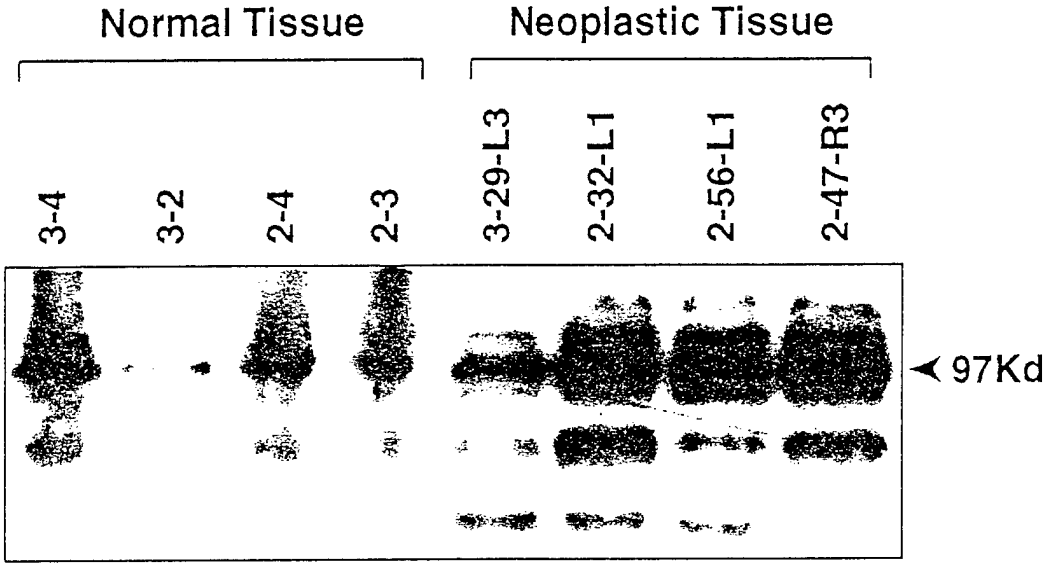


Figure 3: AhR-specific *in situ* Hybridization of Normal Rat Mammary Tissue and DMBA-Induced Mammary Tumors

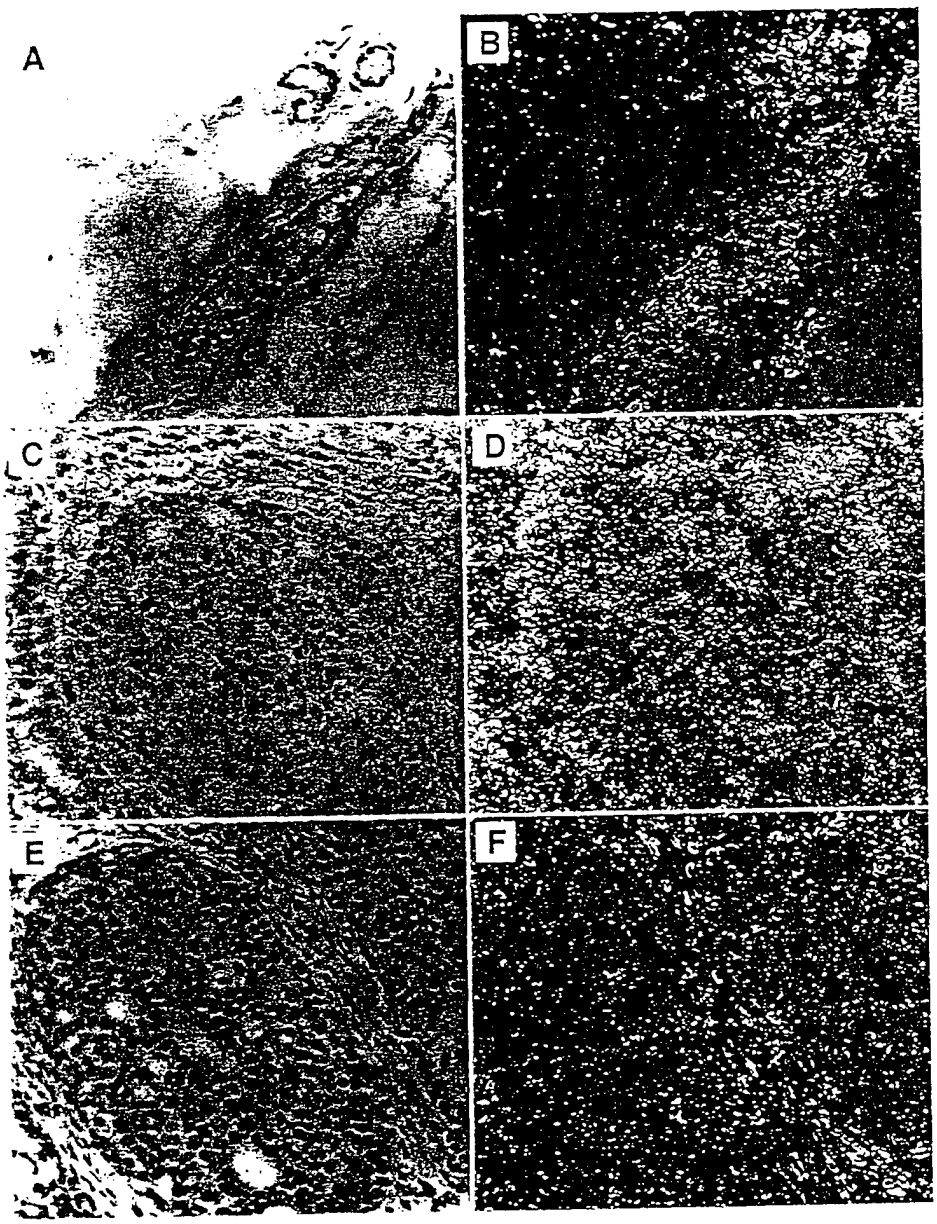


Figure 4: AhR mRNA Expression in Normal Rat Mammary Tissue and DMBA-Induced Mammary Tumors as Assessed by Semi-Quantitative RT-PCR

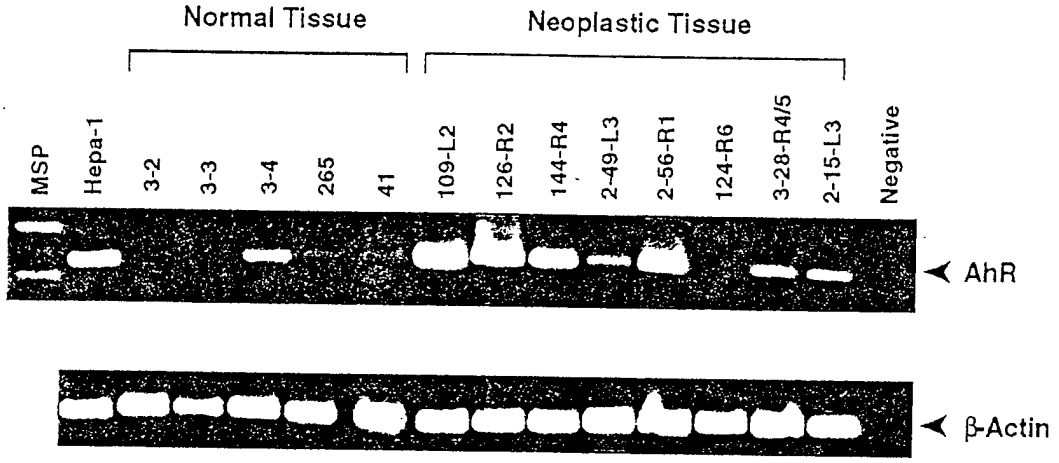


Figure 5: *AhR* mRNA Quantitation by Competitive Mimic RT-PCR

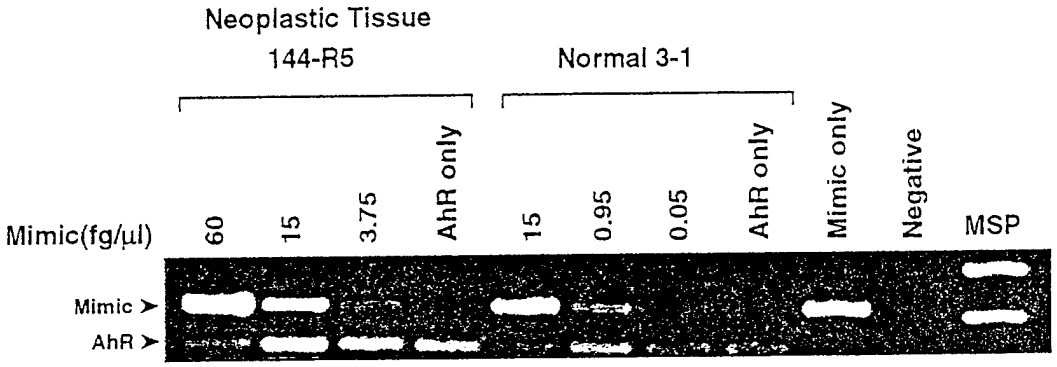


Figure 6: *CYP1A1* and *CYP1B1* mRNA Induction in Mammary Tissue Within Six Hours of DMBA Gavage

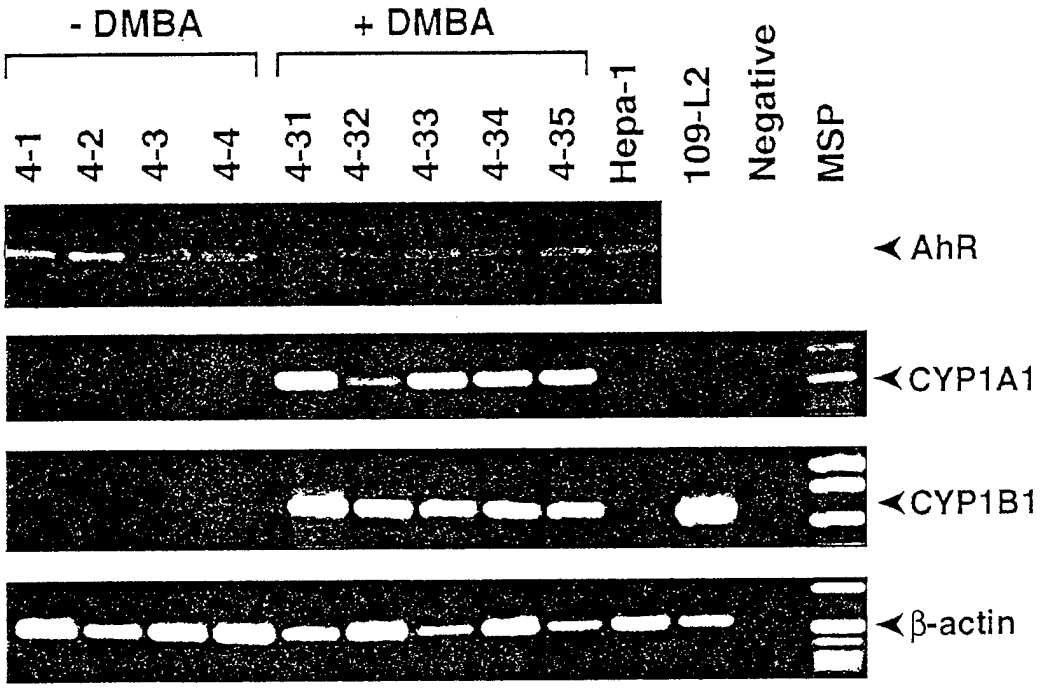
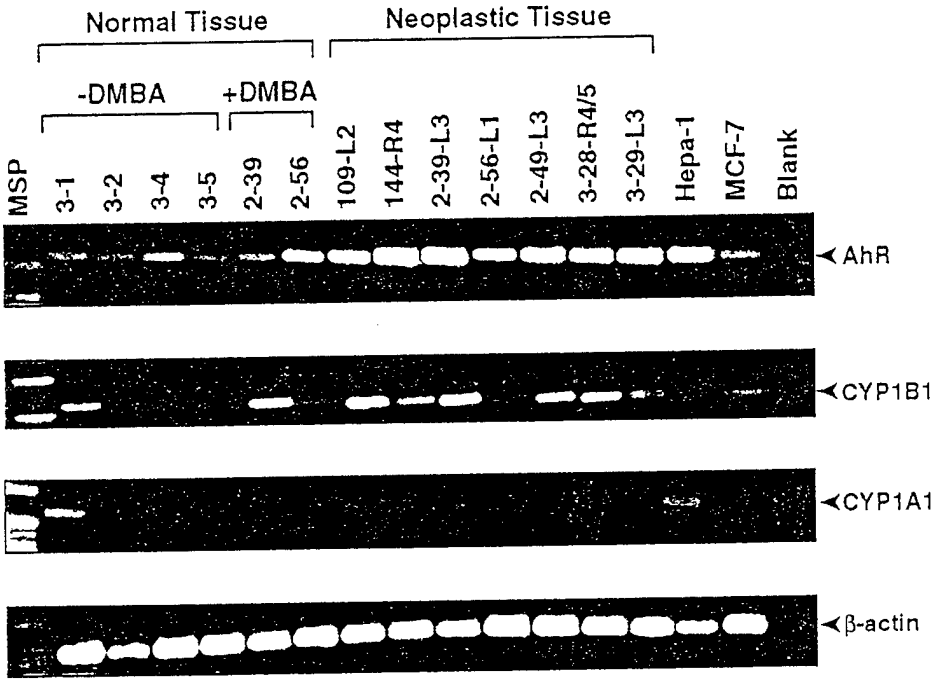


Figure 7: *CYP1A1* and *CYP1B1* mRNA Expression in Normal Rat Mammary Tissue and DMBA-Induced Mammary Tumors



Aberrant Nuclear Factor-κB/Rel Expression and the Pathogenesis of Breast Cancer

Mika A. Sovak,[*§] Robert E. Bellas,[‡§] Dong W. Kim,[‡§] Gregory J. Zanieski,[‡§] Adrianne E. Rogers,[*§] Abdulmaged M. Traish,[‡§] and Gail E. Sonenshein[‡§]

[*]Department of Pathology and Laboratory Medicine, [‡]Department of Biochemistry, and the [§]Program in Research on Women's Health, Boston University School of Medicine, Boston, Massachusetts 02118

Abstract

Expression of nuclear factor-κB (NF-κB)/Rel transcription factors has recently been found to promote cell survival, inhibiting the induction of apoptosis. In most cells other than B lymphocytes, NF-κB/Rel is inactive, sequestered in the cytoplasm. For example, nuclear extracts from two human untransformed breast epithelial cell lines expressed only very low levels of NF-κB. Unexpectedly, nuclear extracts from two human breast tumor cell lines displayed significant levels of NF-κB/Rel. Direct inhibition of this NF-κB/Rel activity in breast cancer cells induced apoptosis. High levels of NF-κB/Rel binding were also observed in carcinogen-induced primary rat mammary tumors, whereas only expectedly low levels were seen in normal rat mammary glands. Furthermore, multiple human breast cancer specimens contained significant levels of nuclear NF-κB/Rel subunits. Thus, aberrant nuclear expression of NF-κB/Rel is associated with breast cancer. Given the role of NF-κB/Rel factors in cell survival, this aberrant activity may play a role in tumor progression, and represents a possible therapeutic target in the treatment of these tumors. (*J. Clin. Invest.* 1997. 100:2952–2960.) Key words: apoptosis • aromatic hydrocarbons • 7,12-dimethylbenz(*a*)anthracene • rat model • transcription factors

Introduction

The incidence of breast cancer has been steadily increasing over the past 50 yr, and is now one of the leading causes of death among American women between the ages of 40–55 (1). In an attempt to find the causes of this increased incidence, both genetic and environmental factors are being studied. Attention has recently focused on the mechanism by which increased exposure to and bioaccumulation of pollutants might have an etiologic role in breast cancer (2–5). The polycyclic aromatic hydrocarbons (PAHs)[1] such as 7,12-dimethylbenz-(*a*)anthracene (DMBA) are specifically of interest with respect to breast cancer (4). The most proximal event in PAH tumorigenesis is the binding of the chemicals to a cytosolic aromatic hydrocarbon receptor (AhR) (6–8). The receptor-ligand complex is translocated to the nucleus where it can bind to and alter the transcriptional activity of DNA that has AhR-responsive elements. One battery of enzymes whose transcriptional induction is a hallmark of DMBA and other PAH exposure is the phase I cytochrome P450 enzymes (9–12). These enzymes aid in the oxidative metabolism of both endogenous substances such as steroids, as well as in the breakdown of exogenous substances such as drugs, chemical carcinogens, and environmental pollutants. The products formed by this oxidative metabolism are often reactive oxygen intermediates. The potential for increased levels of oxidative stress within the cell resultant from exposure to environmental carcinogens led us to hypothesize that this might activate expression of the nuclear factor (NF)-κB/Rel family of transcription factors.

NF-κB/Rel is a family of dimeric transcription factors whose DNA-binding domains have considerable homology with an ~ 300 amino acid region of the v-Rel oncoprotein and was thus termed the Rel homology domain (RHD) (13–15). Classical NF-κB is a heterodimer composed of a 50-kD (p50) and a 65-kD (p65 or RelA) subunit (16). Other members of the mammalian Rel family include c-Rel, p52, and RelB (16–19). The activity of many of these factors is controlled post-translationally by their subcellular localization. In most cells other than mature B lymphocytes, NF-κB/Rel proteins are sequestered as inactive forms in the cytoplasm by association with inhibitory proteins, termed IκB's, for which IκB-α represents the prototype (16, 20–22). Activation involves IκB degradation, and nuclear translocation of the NF-κB/Rel protein. Many signals that activate NF-κB/Rel do so through a final common pathway of increasing cellular oxidative stress (23). Genes regulated by NF-κB/Rel include those involved in immune and inflammatory responses, cellular proliferation, and adhesion molecules (16, 20, 21). More recently NF-κB/Rel has been implicated in control of apoptosis. For example, we have shown that inhibition of constitutive expression of NF-κB/Rel in B cell lymphomas leads to the induction of apoptosis, and that ectopic c-Rel expression promotes cell survival (24, 25). Recently we have obtained similar data with two untransformed hepatocyte cell lines, which express classical NF-κB

Address correspondence to Gail E. Sonenshein, Department of Biochemistry, Boston University Medical School, 80 East Concord Street, Boston, MA 02118-2394. Phone: 617-638-4120; FAX: 617-638-5339; E-mail: gsonensh@acs.bu.edu

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1. *Abbreviations used in this paper:* AhR, Aromatic hydrocarbon receptor; BSAP; B cell specific activator protein; CAT, chloramphenicol acetyl transferase; dm, double mutant; DMBA, 7,12-dimethylbenz(*a*)anthracene; GST, glutathione-S-transferase; IκB-α, NF-κB/Rel inhibitor-α; NF-κB, nuclear factor-κB; PAH, polycyclic aromatic hydrocarbon; URE, upstream regulatory element.

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constitutively (26, 27). Activation of NF-κB/Rel by TNF-α has recently been linked to protection of multiple types of cells from apoptosis (28–31). Here we report that NF-κB/Rel is aberrantly activated in human breast cancer and in rat mammary tumors from the aromatic hydrocarbon-induced model of breast cancer. Inhibition of this activity in human breast cancer cell lines leads to apoptosis. These results suggest an important role for NF-κB/Rel in the pathogenesis of breast cancer and in potential treatment modalities.

Methods

Cell lines. The MCF7 cell line, the prototype of estrogen-dependent breast cancer cells, was established from the pleural effusion of a patient with metastatic adenocarcinoma (32). The 578T tumor cell line was established from a patient with infiltrating ductal carcinoma and does not express estrogen receptors (33); normal breast tissue from this same patient was taken to establish the untransformed breast cell line 578Bst (33). The MCF 10F cell line was established from mammary tissue from a patient with fibrocystic breast disease and is also estrogen receptor negative (34, 35).

Electrophoretic mobility shift analysis. Nuclear extracts for electrophoretic mobility shift analyses (EMSA) were prepared by the method of Dignam et al. (36). URE (5′-GATCCAAGTCCGGGTTTTCCCCAACC-3′; core sequence is underlined) (37), and PU.1 (GATCTACTTCTGCTTTTG, where the core element is underlined) oligonucleotides were end labeled with large Klenow fragment of DNA polymerase and [^{32}P]dNTPs. The electrophoretic mobility shift assay was performed using ~ 2 ng of labeled oligonucleotide (20,000 dpm), 5 μg of nuclear extract, 5 μl of sample buffer (10 mM Hepes, 4 mM dithiothreitol, 0.5% Triton X-100, and 2.5% glycerol), 2.5 μg poly dI-dC as nonspecific competitor and adjusted to 100 mM with KCl in a final volume of 25 μl. This mixture was incubated at room temperature for 30 min. Complexes were resolved in a 4.5% polyacrylamide gel using 0.5× TBE running buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA pH 8.0). Specificity of binding was tested using competition analyses in which 10-fold molar excess of wild-type or double mutant URE (URE 1, see below) was added to a binding reaction. For IκB-α blocking experiments, 1 μg of IκB-α-GST fusion protein was added to the reaction after the 30-min incubation, and the mixture allowed to incubate for an additional 1–2 h at 4°C.

Microinjection analysis. Exponentially growing 578T cells were plated on tissue culture dishes. After 24 h, the medium was supplemented with 20 mM Hepes (pH 7.3) to maintain pH when exposed to open air. All cell nuclei in a defined grid were microinjected at 1.4 psi at a rate of ~ 20 cells per minute as described previously (38). Successful microinjection was estimated to occur > 90% of the time. Purified IκB-α-glutathione-S-transferase (IκB-α-GST), kindly provided by U. Siebenlist, or GST protein was used at 1 μg/μl. Affinity purified antibodies to p65 or c-Rel, SC109 and SC070, respectively (Santa Cruz Biotechnology; Santa Cruz, CA), were used at 4 μg/μl in the absence or presence of 4 μg/μl cognate peptide. Double-stranded oligonucleotides were microinjected at a concentration of 200 ng/μl. Oligonucleotides used were: NF-κB, wild-type element from the immunoglobulin κ light chain enhancer (39); wt URE, see above; UREm1 (5′-GATCCAAGTCCG**CCTTTTCCCC**AACC-3′) and UREm2 (5′-GATCCAAGTCC**GGGTTGGCCCC**AACC-3′), mutant forms of the URE κB element (mutated bases are indicated in bold), which fail to bind NF-κB factors.

Cells were stained with 10 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO), 0.1% Triton X-100, 50 μg/ml RNase A in PBS for 15 min and visualized on a Nikon Optiphot Fluorescence microscope (Nikon Inc., Tokyo, Japan) and fluorescent images recorded at 200× using Kodak Tmax 3200 film (Eastman Kodak Co., Rochester, NY). For trypan blue analysis, cloning rings were placed over the microinjected areas. At various time points after microinjection, the supernatant, containing cells that lost adherence during incubation, were transferred to a 96-well plate for trypan blue analysis. The adherent cells were removed by trypsinization and added to the same well. Trypan blue was added to 0.04% and the plate incubated at 37°C. After 30 min, the percentage of positive staining cells was determined by microscopic visualization at a magnification of 100 under brightfield illumination.

Transfection analysis. 578T and MCF7 cells were transfected using the modified calcium phosphate procedure of Chen and Okayama (40) as we have described previously (41). The total amount of plasmid DNA transfected into the cells was adjusted to 25 μg using either pBlueScript or pUC19 plasmid DNA where necessary. Results were normalized to a TK-luciferase construct and are presented as percent of URE$_2$-TK-CAT or p1.6 Bgl-CAT wt activity (mean±SD), which have been set at 100%. Data shown are representative of three independent experiments.

Rat mammary gland and tumor analysis. Virgin female Sprague-Dawley rats fed AIN76 diet were treated according to a protocol approved by the Boston University Institutional Animal Care and Use Committee. Animals were given a single intragastric dose of 15 mg/kg DMBA at 8 wk of age. Tumors, which were first detected by palpation after 7 wk, were rapidly removed at necropsy after death by CO_2 inhalation. Normal mammary glands, similarly excised from untreated control rats, and tumors were frozen in liquid nitrogen. Animals were killed 16 wk after DMBA treatment at 24 wk of age, except when tumors appeared ulcerated. Samples were pulverized on dry ice using a Bessman tissue pulverizer (Spectrum Industries, Gardena, CA). Frozen tissue powder was homogenized (0.5 grams/ml) in TEGT/MO buffer [50 mM Tris/HCl, 1 mM EDTA, 10% (vol/vol) glycerol, 10 mM monothioglycerol, 10 mM sodium molybdate, pH 7.4 containing 0.02% sodium azide] using a Polytron. After the initial burst proteolytic inhibitors were added to a final concentration of: 0.5 mM PMSF, 1 μg/ml leupeptin, 100 μg/ml aprotinin, 10 μg/ml pepstatin, and 100 μg/ml bacitracin. Homogenates were centrifuged for 10 min at 3,000 rpm at 2°C. Nuclear pellets were extracted via the procedure of Dignam et al. (36) and analyzed as described above.

Human breast cancer specimen analysis. Primary human breast cancer tissue, obtained with IRB approval, was frozen in dry ice and stored frozen until samples were processed for nuclear protein extraction. Nuclear pellets were obtained as described above and washed twice with TEGT/MO buffer and proteins extracted in TEGT/MO buffer plus protease inhibitors adjusted to 0.4 M KCl for 45 min. The debris was removed by centrifugation at 30,000 rpm and the nuclear extract stored frozen. For immunoblot analysis, 20–100 μg of protein were resolved on a 10% polyacrylamide gel and transferred to a 0.45-μm pore PVDF membrane (Millipore). Blots were blocked for 1 h at room temperature in 5% milk (Carnation) in Tris-buffered saline (TBS), then probed with antibody diluted (1:1,000) in 5% milk in TBS for 1 h at room temperature. After three washes in 0.5% NP-40 in TBS, blots were incubated in secondary horseradish peroxidase (HRP)-conjugated goat anti–rabbit antibody at 1:1,000 dilution for 1 h at room temperature. Bands were visualized by chemiluminescence.

Results

Human breast epithelial tumor cell lines constitutively express high levels of functional nuclear NF-κB/Rel activity. To begin to analyze the role of NF-κB/Rel in regulation of epithelial cell proliferation, we performed EMSA on nuclear extracts of untreated, exponentially growing breast tumor and untransformed breast epithelial cell lines to assess basal NF-κB/Rel binding activity. The oligonucleotide containing the NF-κB URE from the c-*myc* gene (37) was used as probe; this oligonucleotide has been shown to bind multiple NF-κB/Rel complexes efficiently (41–44). Significant levels of nuclear NF-κB/Rel complexes were detected in MCF7 (32) and 578T (33) hu-

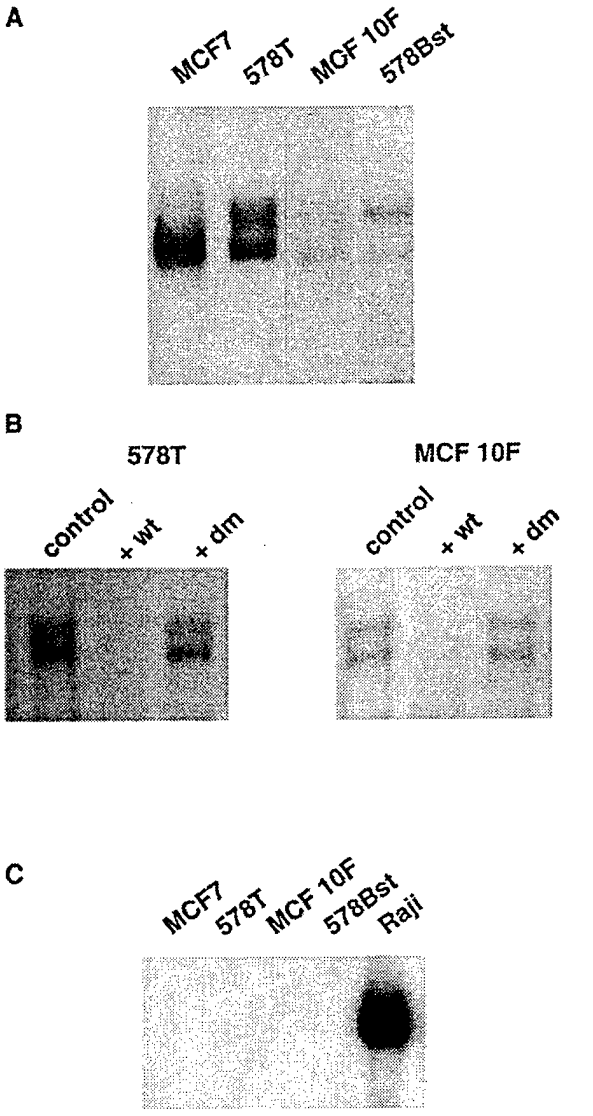

Figure 1. Transformed human breast cancer cell lines constitutively display high levels of nuclear NF-κB/Rel factor binding. (*A*) Nuclear extracts from transformed MCF7 and 578T and untransformed MCF 10F and 578Bst breast epithelial cell lines in exponential growth were used in EMSA analysis with URE NF-κB element oligonucleotide as probe. The level of binding varied amongst the several MCF7 isolates studied, which is in accordance with the recent inability of another group to detect significant levels in their nuclear preparations (70). (*B*) Nuclear extracts from 578T and MCF 10F cells were used in EMSA analysis with the URE NF-κB oligonucleotide as probe in the absence (*control*) or presence of 10-fold molar excess unlabeled wild-type (*wt*) or double mutant URE m1 (*dm*) as competitor. (*C*) EMSA of PU.1 binding was performed, as above. Nuclear extract from the Raji Burkitt lymphoma B cell line was used as a positive control.

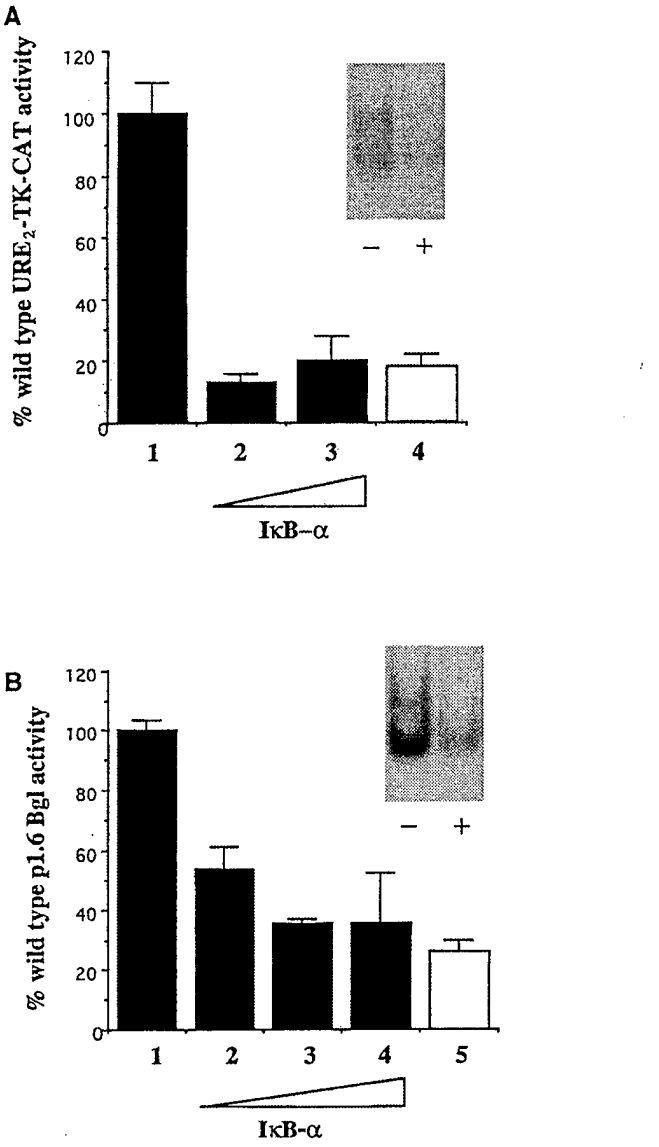

Figure 2. Constitutive nuclear NF-κB/Rel in transformed breast cancer lines is functional. (*A*) 578T cells were transfected in duplicate with either 10 μg of the URE_2-TK-CAT wt reporter plasmid in the absence (bar *1*) or presence of increasing amounts of IκB-α expression vector (3 and 9 μg, bars *2* and *3*, respectively), or with 10 μg of the URE_2-TK-CAT dm plasmid (bar *4*). The total amount of DNA transfected into the cells was adjusted to 25 μg using pBlueScript plasmid DNA. Results were normalized to a TK-luciferase construct and are presented as percentage of URE_2-TK-CAT wt activity (mean±SD), which is set at 100%. (*Inset*) Nuclear extracts from exponentially growing 578T cells were incubated in the absence (−) or presence (+) of 1 μg of IκB-α-GST protein and subjected to EMSA analysis using the URE oligonucleotide as probe. (*B*) MCF7 cells were transfected in duplicate with 13 μg of p1.6 Bgl-CAT wt plasmid in the absence (bar *1*) or presence of 3, 6, or 12 μg of IκB-α expression vector (bars *2*, *3*, or *4*, respectively). Additionally cells were transfected with 13 μg of p1.6 Bgl-CAT dm plasmid containing two G to C conversions in its two identified NF-κB elements (46) (bar *5*). The total amounts of DNA transfected into cells were equalized to 25 μg using pUC19 DNA. The data are presented relative to p1.6 Bgl-CAT wt activity (mean±SD), which is set at 100%. (*Inset*) Nuclear extracts from exponentially growing MCF7 cells were incubated in the absence (−) or presence (+) of 1 μg of IκB-α-GST protein and subjected to EMSA analysis using the URE oligonucleotide as probe.

man breast tumor cell lines, but not in untransformed MCF 10F (34, 35) and 578Bst (33) breast epithelial cell lines (Fig. 1 *A*). This finding was unexpected since nuclear NF-κB/Rel is thought to be restricted primarily to B lymphocytes (16, 21, 39). Nuclear extracts from MCF7 cells displayed several complexes that resolved as a broad lower band and a faint upper band. The ratio and intensity of these two bands varied with different isolates of this line (compare Fig. 1 *A* and Fig. 2 *B*,

and data not shown), which is known to change phenotype in culture. Multiple complexes were also seen with nuclear extracts from 578T cells. Nuclear extracts from the untransformed MCF 10F and 578Bst cell lines displayed only very low levels of NF-κB/Rel binding, as expected. The bands observed with all cell lines tested comigrated with complexes obtained using nuclear extracts from WEHI 231 B lymphoma cells (data not shown), a cell line known to express high levels of NF-κB/Rel (37, 39, 44). Binding specificity of these proteins to URE was confirmed by successful competition with 10-fold excess unlabeled wild-type oligonucleotide, whereas excess unlabeled mutant oligonucleotide essentially had no effect on binding (Fig. 1 *B* and data not shown).

Transient transfection analyses were performed to test functional Rel activity in the tumor lines. The URE_2-TK-CAT wild-type (wt) and URE_2-TK-CAT double mutant (dm) constructs have two copies of the URE NF-κB binding element from the c-*myc* gene in either wild-type or mutant form, respectively, linked to the heterologous thymidine kinase (TK) promoter and the chloramphenicol acetyltransferase reporter gene (CAT) (37). Cultures of 578T cells at 70% confluence were transiently transfected in duplicate (Fig. 2 *A*). The wild-type element vector gave approximately sevenfold higher levels of activity than the double mutant; an average of 9±2-fold higher levels of activity were obtained in six experiments. A similar value of 11±3-fold ($n = 6$) was obtained using MCF7 cells. To further confirm the observed transactivation was indeed due to constitutive NF-κB/Rel activity, cells were cotransfected in duplicate with URE_2-TK-CAT wt and increasing amounts of an IκB-α expression vector (45). A decrease in relative CAT activity of the wild-type construct to that of the double mutant vector was observed upon cotransfection of as little as 3 μg of the IκB-α vector, indicating that the observed activity of the URE_2-TK-CAT wt construct was due to constitutively functional NF-κB/Rel in the cells (Fig. 2 *A*). Similarly, cotransfection of MCF7 cells was performed with a natural promoter construct responsive to NF-κB, the c-*myc* promoter/exon1 p1.6 Bgl-CAT construct (46). Increasing concentrations of the IκB-α expression vector brought the activity of the wild-type construct down to that of the construct with mutated NF-κB elements (Fig. 2 *B*). Consistent with the effects of the inhibitor in transient transfection, addition of IκB-α-GST protein to the binding reactions with nuclear extracts from either transformed cell line abrogated formation of the upper complexes and significantly reduced the level of the more rapidly migrating complex (*insets*, Figs. 2, *A* and *B*). This finding is expected as IκB-α protein has been shown to prevent binding via selective interaction with p65, c-Rel, and RelB (present in slower migrating complexes); inhibition of binding of p50 or p52 appears to require somewhat higher concentrations of inhibitor protein (reviewed in 16). Lastly, the specific activation of NF-κB/Rel binding as opposed to a general elevation in B cell transcription factors was confirmed by EMSA for the Ets transcription factor member PU.1, expressed predominantly in B lymphocytes and myeloid cells (47–49). No PU.1 binding was observed with nuclear extracts from the breast epithelial cell lines, whereas binding of nuclear extracts from the Burkitt lymphoma Raji B cells to the PU.1 oligonucleotide was easily detected (Fig. 1 *C*). Similarly, we failed to detect binding of the B cell–specific activator protein (BSAP) with nuclear extracts from the untransformed or transformed breast epithelial lines (50) (data not shown). Thus, MCF7 and 578T breast cancer cells have selectively activated constitutive nuclear NF-κB/Rel factors.

Inhibition of the constitutive NF-κB/Rel activity in 578T cells induces apoptosis. Recently, we demonstrated that NF-κB/Rel rescues immature B lymphoma cells and hepatocytes from undergoing apoptosis (24–27) and a similar role in cell survival has been demonstrated upon its induction after TNF-α treatment of various cell lines (28–31). If the aberrant NF-κB/Rel expression we detect in breast cancer cells plays a similar role, specific inhibition of its activity should result in cell death. Thus, we used a microinjection strategy to selectively inhibit NF-κB/Rel expression in breast cancer cells. Areas of ~ 4 mm^2 were defined and all 578T breast cancer cells within the grid were microinjected with either the specific NF-κB/Rel inhibitory IκB-α-GST fusion protein or GST protein alone as control. After 3.5 h, the nuclear morphology of the cells was examined by microscopic observation after staining with propidium iodide. Nuclear condensation, a hallmark of apoptosis, was clearly visualized in a field of cells microinjected with IκB-α-GST protein (Fig. 3 *A*, *left bottom panel*), but not with GST protein or in noninjected cells (Fig. 3 *A*, *top panels*). Typical cells with condensed chromatin after microinjection of IκB-α-GST are shown in Fig. 3 *A* (*right bottom panel*). A marked drop in cell density with time was noted after microinjection with IκB-α-GST protein but not control GST protein, possibly reflective of the observation that cells undergoing apoptosis detach and float off the dish surface.

To obtain more quantitative analysis, trypan blue staining was used as a measure of cell viability after microinjection with IκB-α-GST or GST protein, as above, or with affinity purified antibodies to either the p65 or c-Rel subunit. We noted that the adherent cell density was reduced by $\sim$ 50–60% after injection with IκB-α-GST (Fig. 3 *A*), whereas only 4–5% of the cell population injected with GST protein alone lost adherence (data not shown). Therefore, nonadherent and adherent cells were combined and analyzed for loss of cell viability 20–22 h after microinjection (Fig. 3 *B*). 20% of the cells microinjected with IκB-α-GST stained positive for trypan blue, compared to $< 3\%$ of cells microinjected with GST or nonmicroinjected cells. Thus, IκB-α–mediated inhibition of NF-κB/Rel caused death of 578T breast cancer cells by apoptosis. In a preliminary supershift EMSA analysis, 578T cells were found to express significant levels of p65 whereas only low levels of c-Rel subunits were detected (data not shown). Microinjection with an affinity purified antibody to p65 induced cell death in $\sim$ 45% of 578T cells within 20–22 h, which was inhibited by preincubation of the antibody with 40-fold molar excess of its cognate peptide (Fig. 3 *B*). In contrast, microinjection of the antibody against c-Rel induced only modest additional loss of cell viability over background levels seen with microinjection with GST protein alone.

To further evaluate the effects of specific inhibition of NF-κB/Rel activity, we also monitored loss of cell viability after microinjection of double-stranded (ds) oligonucleotides harboring either wild-type or mutated NF-κB elements. Recent studies have demonstrated that microinjection of ds oligonucleotides containing a transcription factor binding site can compete in vivo and inhibit the activity of the factor (51, 52). Microinjection of oligonucleotides containing the wild-type element from the κ light chain enhancer (κB) or from the c-*myc* gene (URE) induced significant levels of apoptosis within 20 h (Fig. 3 *C*). In contrast, microinjection of oligonucleotides con-

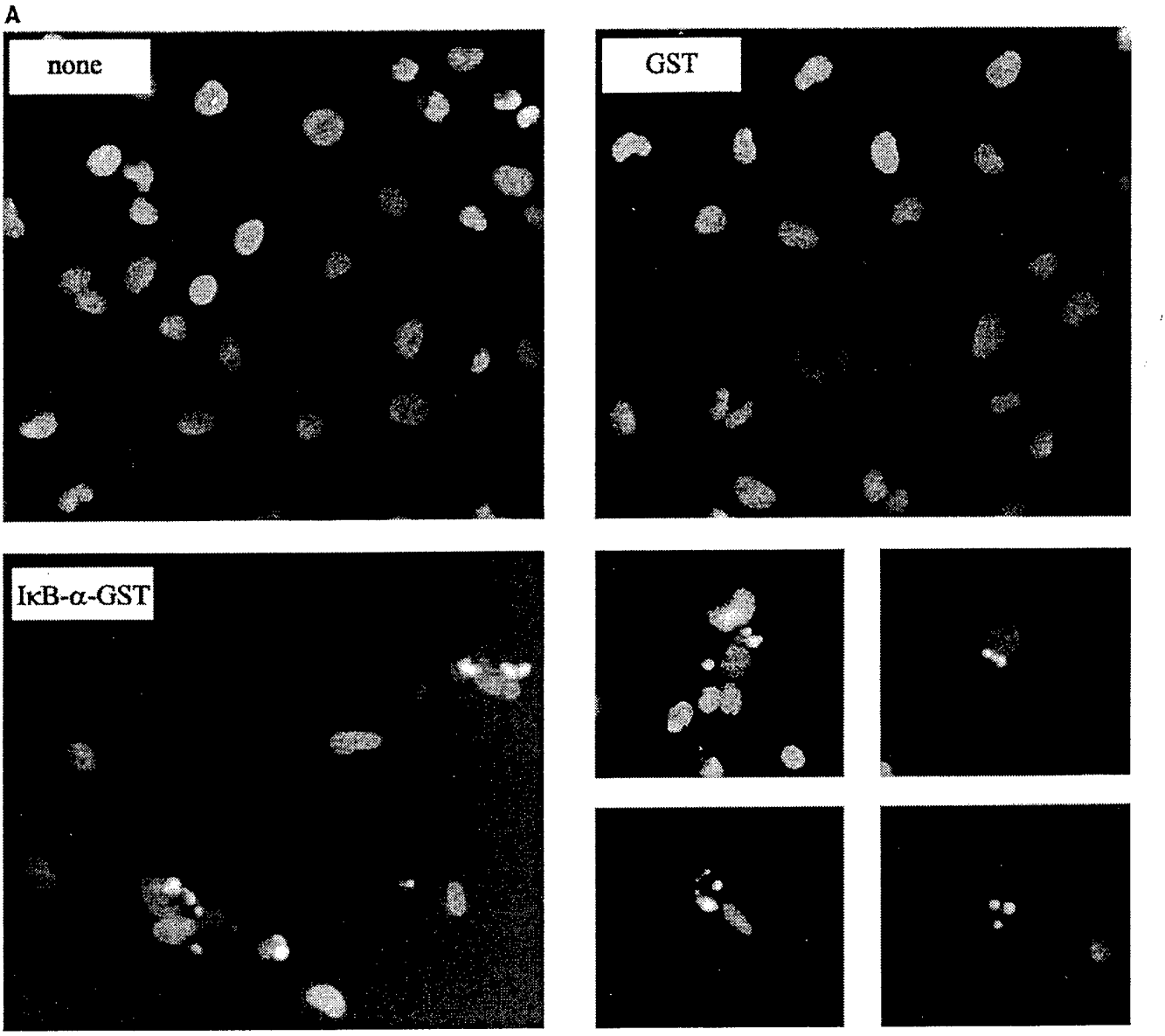
A
none
GST
IκB-α-GST
B
none
GST
IκB-α-GST
α–p65
α–p65 +peptide
α–c-rel
α–c-rel +peptide
0
10
20
30
40
50
per cent trypan blue positive
C
none
NF-κB
URE
UREm1
UREm2
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Figure 3

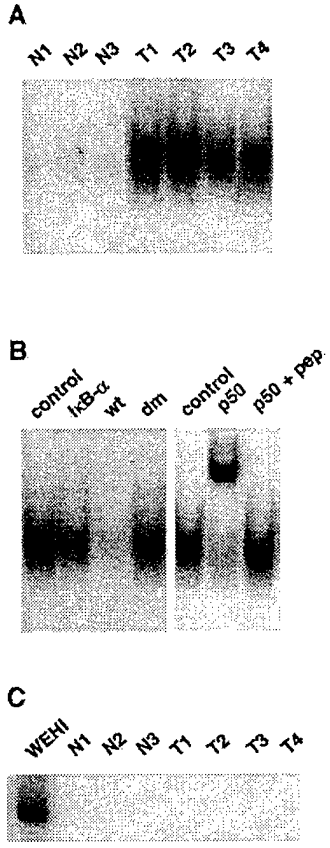

Figure 4. Nuclear expression of NF-κB/Rel in primary rat mammary tumors but not normal mammary gland. (*A*) Nuclear extracts were prepared from mammary glands from three untreated control rats (*N1–N3*) or from mammary tumors from DMBA-treated rats (*T1–T4*) and equal amounts (5 μg) subjected to EMSA for binding to the URE oligonucleotide, as described above. (*B*) EMSA was performed with a nuclear extract from tumor T1 in the absence (*control*) or presence of 1 μg IκB-α-GST protein (IκBα), 50× molar excess unlabeled oligonucleotide containing URE wild type (*wt*) or mutant (*dm*) sequences (*left*). Alternatively, the antibody to the p50 subunit in the absence (*p50*) or presence of cognate peptide (*p50 + pep*) was added to the binding reaction. (*C*) EMSA was performed with nuclear extracts described in part A with the PU.1 oligonucleotide. WEHI 231 B cell nuclear extracts were used as a positive control.

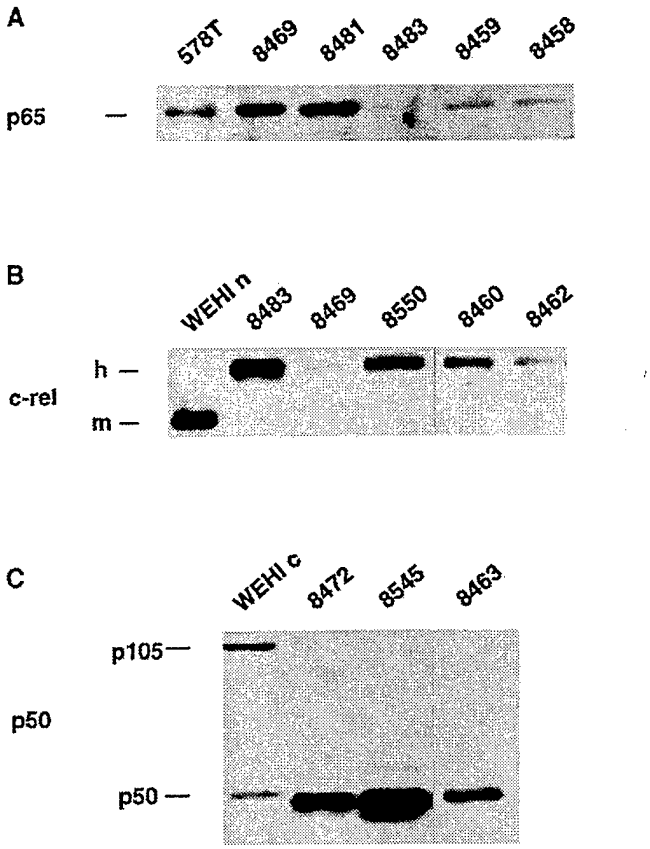

Figure 5. Nuclear expression of NF-κB/Rel subunits in primary human breast cancer tissue. (*A*) p65: Samples of nuclear extracts from multiple tumor specimens (100 μg/lane) were analyzed using the anti-p65 antibody #1226. (*B*) c-Rel: immunoblot analysis was performed on nuclear extracts (50 μg/sample) using an antibody against c-Rel protein (SC070; Santa Cruz Biotechnology Co.). (*C*) p50: immunoblot analysis was performed on nuclear extracts (50 μg/sample) using an antibody against p50 protein (SC114; Santa Cruz Biotechnology Co.). Patient samples are indicated by four digit numbers. As controls for detection, a nuclear extract from exponentially growing 578T cells (20 μg) was used in panel A, and nuclear (*n*) or cytoplasmic (*c*) extracts (50 μg) from WEHI 231 murine B cells (*WEHI*) were used in panels *B* and *C*, respectively. *h*, Human; *m*, mouse.

taining two mutations of the URE (UREm1 and UREm2) that prevent binding of NF-κB/Rel factor did not significantly increase cell death above background levels seen with uninjected cells. In summary, selective inhibition of NF-κB/Rel activity by these three different strategies resulted in apoptosis of 578T breast cancer cells.

DMBA-induced rat mammary tumors express high levels of nuclear NF-κB/Rel binding activity. To verify that the observed nuclear expression of NF-κB/Rel factor complexes in the breast cancer cell lines was not the result of in vitro cell culturing, we have extended these observations to a widely used in vivo rat model of breast cancer. Treatment of female Sprague-Dawley rats with a single dose of the polycyclic aromatic hydrocarbon DMBA results in the induction of mammary gland (breast) tumors within 7–20 wk (53). These tumors are generally well differentiated and retain their hormonal responsiveness. Nuclear extracts were prepared from multiple DMBA-induced rat mammary tumors and from normal mammary glands from untreated rats as controls. High levels of binding to the URE NF-κB oligonucleotide, essentially comparable to that seen with WEHI 231 B cells, were obtained with nuclear extracts from 86% of the 35 tumor specimens analyzed (Fig. 4 *A*, lanes *T1–T4* and data not shown). In contrast, no significant levels of NF-κB/Rel binding were observed with nuclear extracts from the normal mammary gland, as expected (Fig. 4 *A*, lanes *N1–N3* and data not shown). The specificity of the NF-κB/Rel binding was confirmed by competition with excess unlabeled oligonucleotides containing wild-type but not mutant NF-κB elements and the ability of an antibody against the

Figure 3. Microinjection of 578T cells with IκB-α protein induces apoptosis. (*A*) Propidium iodide staining of 578T cells in exponential growth (*none*), and 3.5 h after microinjection with 1 μg/μl GST protein alone or 1 μg/μl IκB-α-GST fusion protein. All cells shown within these representative fields were microinjected. (The left bottom panel displays a representative region of the full field; however, since the numbers of cells microinjected with IκB-α protein were greatly reduced, individual clusters of cells are shown in the smaller panels.) (*B*) 578T cells (between 76 and 141 cells per sample) were microinjected in duplicate and cells stained with trypan blue 20–22 h after microinjection to assess cell viability. Data are expressed as mean±SD and are representative of two experiments. (*C*) 578T cells (between 84 and 164 cells per sample) were microinjected in duplicate with the indicated double stranded wild-type or mutant NF-κB oligonucleotide, and analyzed for trypan blue staining 20 h after treatment. Data are expressed as mean±SD.

p50 subunit and addition of IκB-α-GST protein to significantly ablate binding activity (Fig. 4 *B*). No binding to the PU.1 oligonucleotide was seen with nuclear extracts from either normal mammary gland or mammary tumors (Fig. 4 *C*). These results indicate that the observed NF-κB/Rel binding is not due to contaminating B cells. Thus, NF-κB/Rel expression is selectively induced in vivo within rat mammary gland tumors but is not present normally in mammary glands, suggesting a significant association between the activation of NF-κB/Rel expression and mammary tumor formation.

Nuclei of primary human breast tumor specimens contain NF-κB/Rel subunits. To determine whether NF-κB/Rel factors are constitutively expressed in nuclei of primary human breast cancer tissue, samples procured after surgical removal of breast tumors were analyzed for potential NF-κB/Rel subunit expression by immunoblotting. Nuclear extracts from 13 patient tumor samples were tested with an antibody against the p65 subunit (#1226 [54], kindly provided by N. Rice, National Cancer Institute, Frederick, MD) (Fig. 5 *A* and data not shown). Eight tumors had detectable levels of a band that comigrated with the p65 subunit derived from 578T cells. A second antibody against p65 (SC372; Santa Cruz Biotechnology) was used with its cognate peptide to confirm the specificity of the positive signal seen with the tumor samples (data not shown). Detection of p65 in the nuclear extracts is not due to contamination with cytoplasmic proteins as nuclear extracts from eight tumors were tested and found negative for the cytoplasmic IκB-α protein (data not shown). Overall, nuclear extracts from 15 of 23 specimens displayed positive staining for p65: 8 of 13 specimens were found positive using the #1226 antibody (and four confirmed with SC372) and 7 of 10 specimens were positive using the SC372 antibody (Table I).

Expression of the c-Rel subunit was also assessed by immunoblotting (Fig. 5 *B*). A band that migrated marginally slower than the mouse c-Rel protein was detected with antibody SC070 (Santa Cruz Biotechnology), which was competed away upon addition of cognate peptide (data not shown). Of 23 specimens tested, 20 were found to contain nuclear c-Rel protein (Fig. 5 *B* and data not shown); the level of this expression was in many cases comparable to that seen in WEHI 231 cells, which express very high levels of this subunit (37, 39). In addition, nuclear extracts from tumors contained significant levels of the p50 subunit (Fig. 5 *C* and data not shown), which was shown to be specific by successful competition with cognate peptide (data not shown). Overall, 19 of 21 tumors were positive for this subunit. The absence of detectable p105 precursor, as seen in the cytoplasmic extracts of WEHI 231 B cells (WEHI c), further confirms the absence of cytoplasmic contamination of these nuclear extracts (Fig. 5 *C*). The results, summarized in Table I, indicate that most primary human breast cancer tissue samples express the transactivating p65 and/or c-Rel subunits of NF-κB/Rel. Some samples were negative for both subunits; however, a more complete analysis of breast tumors for all of the NF-κB/Rel subunits may reveal the presence of additional subunits. Preliminary EMSA, which is more sensitive than immunoblotting, has confirmed variable levels of NF-κB/Rel binding in multiple human patient samples (data not shown). Overall, these findings indicate that the nuclear localization of NF-κB/Rel expression is a characteristic of human breast tumor cell lines, DMBA-induced rat mammary tumors, and primary human breast cancer tissue.

Table I. Immunoblot Analysis of Nuclear NF-κB/Rel Subunit Expression in Human Breast Cancer Tissues

Code*	p65	c-Rel	p50
8445	+	+	+
8446	–	+	+
8448	+	+	+
8450	+	+	+
8458	+	+	+
8459	+	+	+
8460	+	+	+
8462	+	+	–
8463	–	+	+
8469	+	+	+
8470	–	+	+
8471	+	+	+
8472	–	+	+
8479	–	–	NA‡
8481	+	+	–
8483	–	+	+
8484	+	+	+
8487	+	+	+
8488	+	+	+
8545	–	+	+
8547	+	–	+
8549	–	–	NA‡
8550	+	+	+
Total positive	15/23	20/23	19/21

*Patient samples are indicated by four digit numbers. ‡Not available.

Discussion

We have analyzed three different systems of breast cancer: in vitro human breast cancer cell lines, an in vivo rat model of breast cancer, and human tissue samples from primary breast tumors. All three have revealed an association between activated NF-κB/Rel and breast cancer. Direct inhibition of the functional NF-κB/Rel factors expressed in 578T cells by microinjection of IκB-α protein, an antibody to the p65 subunit, or ds oligonucleotides containing NF-κB elements led to induction of death of these cells via apoptosis. This study extends our recent work demonstrating that inhibition of the normal constitutive NF-κB/Rel activity in B cell lymphomas leads to cell death (24, 25), to breast cancer where NF-κB/Rel has been aberrantly activated. Our findings suggest that down-regulation of NF-κB/Rel may be useful in the treatment of this disease. Many antioxidants such as pentoxifylline (55) and *N*-acetyl cysteine (56), which are already in clinical use, have been found to repress NF-κB/Rel activity. Preliminary evidence suggests that these agents have significant inhibitory effects on proliferation of breast cancer cells in culture (unpublished observations). Thus, while the efficacy of a specific agent may depend on the nature of the NF-κB/Rel and IκB subunits expressed, our studies suggest NF-κB/Rel is a novel therapeutic target for the treatment of breast cancer.

There has been a steady increase in the incidence of breast cancer over the last several decades, and it has been proposed that this may reflect an increased exposure to environmental

carcinogens such as DMBA (5, 57, 58). Our study of DMBA-induced tumors in rats shows an association between DMBA exposure, breast tumor formation, and activation of NF-κB/Rel. It has been proposed that carcinogens promote tumor progression through DNA damage. The activation of NF-κB/Rel in the tumors, which would promote cell survival, suggests an additional indirect effect of this carcinogen on tumor progression. The mechanism of NF-κB/Rel activation by DMBA remains to be determined. As discussed above, however, a hallmark of DMBA exposure is an increase in P450 enzyme levels with a resultant increase in oxidative stress, which is known to activate NF-κB/Rel (11, 23, 59).

We have recently reported that ectopic expression of c-Rel in WEHI 231 B cells leads to extensive protection from apoptosis induced by engagement of surface immunoglobulin, treatment with TGF-β1, or addition of the protease inhibitor TPCK (24, 25). Ectopic c-Rel expression plays a similar role in protection of hepatocytes from TGF-β1–induced cell death (26). Furthermore, inhibition of endogenous p50/p65 NF-κB activity within these hepatocytes leads to cell death via apoptosis (27). Thus, the finding that many of the breast cancer specimens express c-Rel and p65 suggests that NF-κB/Rel factors play a similar role as a survival gene in these tumors. The primary target(s) of these factors remain a major question. Candidate genes include the known antiapoptotic genes, Bcl-2 and Bcl-X_L. One additional candidate for target is the c-*myc* gene, which is extensively regulated through its two NF-κB/Rel elements (42, 44, 46). Recently, we have shown that ectopic c-*myc* expression protects against apoptosis of B cells (60). Interestingly, studies on patient material and transgenic mice have implicated overexpression of the c-*myc* gene in the etiology of breast cancer (61–66). In human breast cancer, analysis of tumors shows an association between overexpression of the c-*myc* gene and poor prognosis, especially in node-negative patients (61, 62, 67, 68). These findings raise the possibility that the activation of c-*myc* gene by NF-κB may serve to promote both tumor cell protection from apoptosis as well as neoplastic transformation.

With the expanded use of sensitive mammographic and tissue sample screening procedures, there has been a significant rise in detection of potentially premalignant lesions. The prevalence of benign breast conditions or fibrocystic changes is estimated at > 85% in women in the U.S. Approximately one-third of U.S. women undergo breast biopsies; the vast majority of these biopsies show benign disease that are not associated with an increased risk of cancer (69). The quest for markers to positively identify which of these lesions will progress to malignancy is critical to help develop strategies for treatment of benign breast disease and to improve early detection of breast cancer. Nuclear NF-κB/Rel expression is associated with neoplastic breast tissue in humans and laboratory rats. Studies are underway to elucidate any association between the stages of neoplastic transformation and expression of NF-κB/Rel factors as markers of disease.

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Estrogen and Progesterone Receptor Concentrations and Prevalence of Tumor Hormonal Phenotypes in Older Breast Cancer Patients

Jacqueline Ashba, M.P.H., and Abdulmaged M. Traish, Ph.D.

Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts

Address all correspondence and reprint requests to: A. Traish, Ph.D., Department of Biochemistry, Boston University School of Medicine, 80 East Concord St., Boston, MA 02118.

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ABSTRACT: We examined the concentrations of estrogen (ER) and progesterone receptors (PR) and the distribution of tumor phenotypes as a function of age in breast cancer patients. ER and PR concentrations were determined in tissue biopsies from 1739 patients with primary breast cancer, using ligand binding assays. Tumors were classified as estrogen receptor positive (ER+) or negative (ER–) and progesterone receptor positive (PR+) or negative (PR–) based on the presence or absence of receptor binding activity. Tumors were stratified into four phenotypes: ER+PR+; ER+PR–; ER–PR+; and ER–PR–. Significant positive associations were found between ER concentration and age ($p = 0.0001$) and between PR concentration and age ($p = 0.0002$). The median ER concentrations were statistically different by age groups, with the greatest levels in older versus younger patients. The prevalence of ER+PR+ tumor phenotype increased with age. In contrast, the prevalence of ER–PR– and ER–PR+ tumor phenotypes decreased with age. The median PR-to-ER ratio decreased with age ($p = 0.0001$), and this trend was attributed to increased ER concentration with age. The prevalence of ER–PR– and ER–PR+ tumor phenotypes is greater in younger patients suggesting that hormonal regulation of ER gene expression may be responsible for the observed age disparity of tumor phenotypes in breast cancer.

KEY WORDS: breast cancer, estrogen receptors, progesterone receptors, risk factors.

I. INTRODUCTION

Breast cancer is the second leading cause of cancer death among women in the United States. The incidence of breast cancer has been increasing since the 1980s, with the highest incidence occurring in postmenopausal women.[1,2] It has been well documented that ovarian hormones play a major role in the growth of breast cancer. Tumor expression of ER and PR is associated with favorable response to endocrine therapy,[3,4] survival,[5–7] and histological differentiation,[8–10] and is associated with certain risk factors for breast cancer.[11,12] Of all the risk factors studied, age is a strong independent predictor of estrogen receptor levels and breast cancer incidence.[12]

Most studies on the effects of age on hormone receptors have examined mainly the presence or absence of ER and PR,[13–16] and only limited studies examined the relationship between age and ER and PR concentrations.[17,18] Furthermore, the paucity of data regarding the effects of age on tumor hormonal phenotypes in breast cancer suggests the need for better understanding of the relationship between age and tumor hormonal responsiveness.

In this study, we have examined the relationship between age and ER and PR concentrations in a sample of hospital-based breast cancer patients. Since the combination of ER and PR status is thought to characterize tumors with different clinical outcomes,[17] we also analyzed the relationship between age and tumor phenotype. This study sample included a substantial representation of patients beyond the seventh decade of life. Further, we used data generated by ligand binding assays, which unlike the enzyme immunoassay (EIA) or immunocytochemical assays, can distinguish between functional and nonfunctional hor-

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mone receptors. Also, all the data were collected in a single laboratory using one standard assay method, thereby reducing data variations when compared with data gathered from several different laboratories using different assay methods. The objective of this study was to describe the ER and PR concentrations and the distribution of tumor phenotypes as a function of age in breast cancer patients. Together with other prognostic factors, the concentrations of ER and PR within a given tumor phenotype may serve as a better guide for the appropriate therapeutic modality in the treatment of breast cancer patients.

II. MATERIALS AND METHODS

A. Patient Population

Human breast tumors from 1739 patients were assayed for ER and PR content at Boston University School of Medicine Hormone Assay Laboratory, between December 1988 and December 1993. All data were obtained from primary breast cancer patients undergoing surgery for treatment of breast cancer in several Boston area hospitals. The Institutional Review Board of Boston University School of Medicine approved this study.

B. Estrogen and Progesterone Receptor Assays and Tumor Hormonal Phenotypes

The concentration of unoccupied ER and PR were determined by ligand binding analysis as described previously.[19] [^{3}H]Estradiol was used as a ligand for ER and [^{3}H] ORG 2058 was used as ligand for PR.[19] The binding data were normalized as femtomoles of specifically bound ligand per milligram (fmol/mg) of cytosol proteins. We have chosen cutoff values for ER of 10 fmol/mg protein, and for PR of 2 fmol/mg protein since these values were reported to the American College of Pathologists (CAP) as part of our laboratory certification. These cutoff values were chosen because they reflect the sensitivity of the assays using ligands with high specific activity. Tumors were stratified into four groups according to their ER and PR status: (i) ER+PR+; (ii) ER−PR−; (iii) ER+PR−; and (iv) ER−PR+. Others using ligand binding and gel shift assay reported similar stratification.[20]

C. Statistical Analyses

Patients were stratified into five age groups: Group I, less than 45 years; Group II, 45–54 years; Group III, 55–64 years; Group IV, 65–74 years; and Group V, >74 years. The choice of this breakdown in age was chosen to reflect the clinical application of endocrine therapy and other risk factors and possible linkage to clinical trials of breast cancer. Separate linear regression analyses were performed to examine the association between age and ER and PR concentrations. In addition, for each age group linear regression analyses were performed between ER and PR concentrations and age. Multiple linear regression analyses were used to test the possibility of a nonlinear function of hormone receptor levels and age, by adding (age)2 and (age)3 to the model. A test for normality of the distribution of ER and PR levels was performed. A nonparametric test (Kruskal-Wallis test) was used to compare the median values of ER and PR by age groups. Chi-square analysis was used to examine differences in age and the combined ER and PR status of tumors. For tumors with ER+PR+ phenotype, the ratio of PR to ER was calculated for each age group and the general linear model was used to compare the median PR-to-ER ratios, as well as the median ER and PR levels by age group. All statistical analyses were performed using mainframe SAS version 6.10 at the Boston University Medical Center.

III. RESULTS

Estrogen and progesterone receptor concentrations were examined in tumors from 1739 breast cancer patients, who underwent surgery for treatment of breast cancer. The mean age of patients was 64 (range: 18–106 years). There were 219 patients (13%) younger than 45 years of age; 266 patients (15%) between ages 45 and 54 years; 330 patients (19%) between ages 55 and 64 years; 469 patients (27%) between ages 65 and 74 years; and 455 (26%) older than the age of 74 years.

Simple linear regression analysis of ER and PR concentrations with age showed a positive association between ER and age ($p = 0.0001$; $r = 0.26$; slope = 2.90) and PR and age ($p = 0.0002$; $r = 0.09$; slope = 1.77). The possibility of a nonlinear relationship between age and either ER or PR was tested; however, we did not find statistically significant associa-

tions by multiple linear regression analysis. Univariate analysis of the distribution of ER and PR concentrations did not show evidence of normality; therefore, we used the median values for ER and PR and PR-to-ER ratio rather than the means in all statistical comparisons. When analyzed by the Kruskal-Wallis test, the median ER increased with age (Table I), and the medians were statistically different across age groups ($\chi^2 = 156.7$; $p = 0.0001$). Similarly, the median values for PR were statistically significant across age groups ($\chi^2 = 18.86$; $p = 0.0008$). However, the patterns were different between these two receptors across age groups (Table I). Chi-square analyses showed statistical significance when age group was cross-tabulated with tumor phenotype ($\chi^2 = 67.4$; $p = 0.001$). Overall, ER+PR+ (1200/1739) phenotype was the most prevalent (69%), followed by ER−PR+ (338/1739) or (19%); 7% were ER−PR− and 5% were ER+PR−.

The prevalence of ER+PR+ tumor phenotype increased with age, approaching 80% in the >74-year age group (Figure 1). Tumor phenotypes exhibiting ER−PR+ status (Figure 2) and ER−PR− tumors (Figure 3) showed a decreasing trend in age group-specific prevalence. However, tumor phenotypes with ER+PR− showed no consistent trend with age (Figure 4).

It is well recognized that PR synthesis is under estrogen regulation. For this reason, we examined the effects of age on PR expression. The ratio of PR to ER was calculated as a function of age only for the ER+PR+ tumors (1200 subjects). Regression analysis showed a negative association between the PR-to-ER ratio and age ($p = 0.0001$; $r = 0.21$; slope = −0.06). The median PR-to-ER ratios varied markedly across age groups (Table II) and were statistically different ($\chi^2 = 106.32$; $p = 0.0001$). For ER+PR+ tumors (Table II), the median ER and PR concentrations were compared across age groups. A linear trend was observed; median ER concentrations increased with age and were statistically different across age groups ($\chi^2 = 129.9$; $p = 0.0001$). Comparisons of median PR levels by age group did not show a linear trend and were not statistically different across age groups ($\chi^2 = 6.82$; $p = 0.1457$).

TABLE I
Median ER and PR Concentrations (fmol/mg) by Age Group for All Tumors (N = 1739)

Age group (years)	N	Median ER	Median PR
<45	219	14	31
45–54	266	19	39
55–64	330	37	25.5
65–74	469	62	36
>74	455	92	54

Note: Kruskal-Wallis chi-square = 156.73; $p = 0.0001$ for median ER values. Kruskal-Wallis chi-square = 18.85; *$p = 0.008$ for median PR values.*

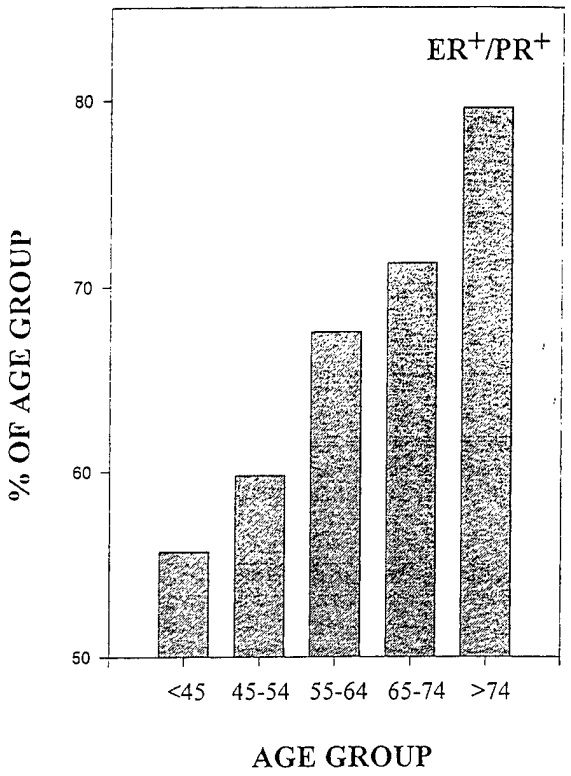

FIGURE 1. Prevalence of ER+PR+ tumor phenotype in breast cancer patients of various age groups. ER and PR we measured in cytosols as described in Materials and Methods. The patients were stratified by age into five age groups, and the percentage of age groups with ER+PR+ tumors was plotted as function of age group (N = 1200 patients).

IV. DISCUSSION

Estrogen (ER) and progesterone receptors (PR) status, among other biochemical tumor markers, were deemed useful prognostic indices in metastatic breast

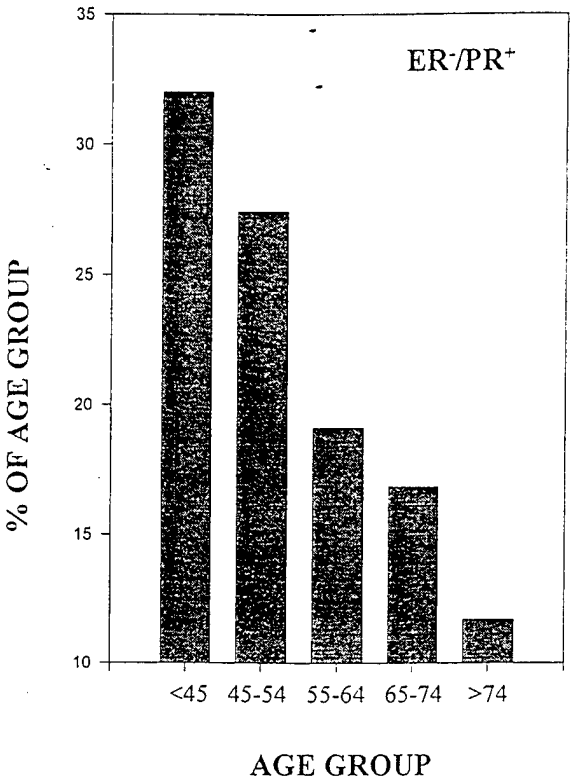

FIGURE 2. Prevalence of ER–PR+ tumor phenotype in breast cancer patients of various age groups. ER and PR we measured in cytosols as described in Materials and Methods. The patients were stratified by age into five age groups, and the percentage of age groups with ER–PR+ tumors was plotted as function of age group (N = 338 patients).

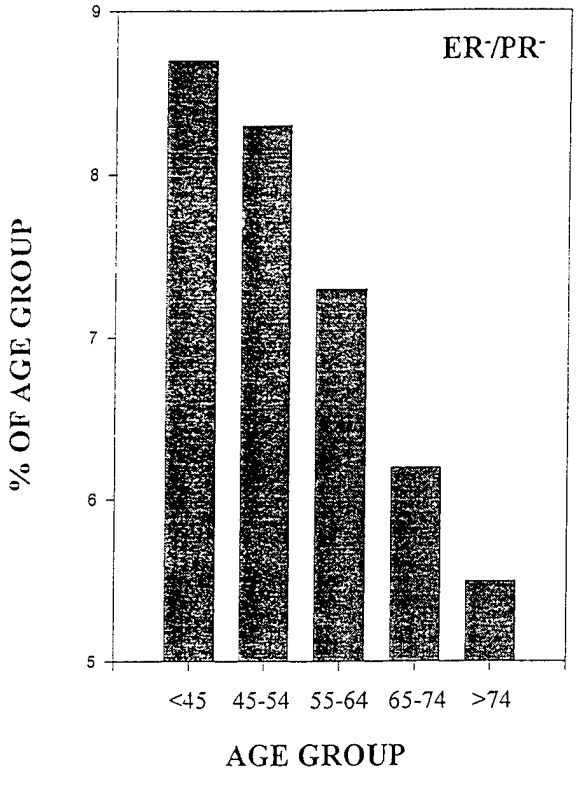

FIGURE 3. Prevalence of ER–PR– tumor phenotype in breast cancer patients of various age groups. ER and PR we measured in cytosols as described in Materials and Methods. The patients were stratified by age into five age groups, and the percentage of age groups with ER–PR– tumors was plotted as function of age group (N = 119).

cancer. Continued expression of these receptors in human breast tumors is associated with disease-free survival. ER and PR are detected in approximately 50–80% of all breast tumors and their expression is of clinical significance for selection of patients who are likely to respond to antiestrogen therapy. The goal of this study was to investigate the effects of age on the concentrations and status of ER and PR in human breast tumors. Evidence is presented that changes in ER and PR concentrations, as well as the prevalence of tumor hormonal phenotypes, are significantly associated with age. These observations are in agreement with previous reports.[17,18,21–23] The median ER level was greater in older patients compared with younger patients, suggesting changes in the regulation of ER expression in young patients. One possibility to explain the increased ER levels in tumors of older patients is the availability of unoccupied ER binding sites due to lower levels of circulating estrogens in postmenopausal patients. Alternatively, ER expression is upregulated in older patients due to lower circulating endocrine hormones. The reduced levels of ER in tumors from younger patients, however, may be attributed in part to estrogen receptor occupancy by endogenous estrogens and/or to downregulation of ER expression by progestins and other endocrine hormones.

PR concentrations decreased with age for patients between 45 and 54 years of age, followed by an increase in PR with age above 54 years. The point of inflection of the median PR concentration at the 45 to 54-year age group may reflect biological changes occurring in the perimenopausal years, as reported previously.[17,18] We speculate that breast cancer may be a disease with a dichotomous nature represented by hormonal insensitive tumors in the premenopausal patients and hormone dependent tumors in the postmenopausal patients.

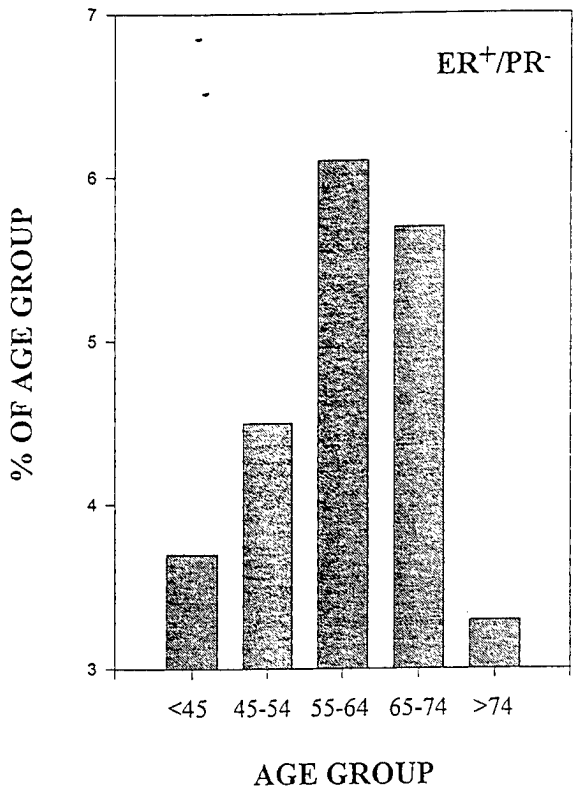

FIGURE 4. Prevalence of ER+PR– tumor phenotype in breast cancer patients of various age groups. ER and PR we measured in cytosols as described in Materials and Methods. The patients were stratified by age into five age groups, and the percentage of age groups with ER+PR– tumors was plotted as function of age group (N = 82 patients).

Normal breast cells express reduced concentrations of ER and PR as compared with breast cancer cells. The transformation of normal cells into neoplastic cells may lead to retention or loss of expression of regulatory proteins such as ER and PR. The ability of tumor cells to retain expression of regulatory proteins reflects the state of tumor differentiation.[10,24–28] Thus, during tumor growth and progression, four tumor hormonal phenotypes may emerge. Tumor cells that retain ER and PR expression remain ER+ and PR+ and are expected to remain differentiated. Tumor progression may lead to loss of expression of ER and PR leading to an ER– and PR– phenotype with some dedifferentiation. Recently, we have demonstrated loss of expression of a novel nuclear protein in tumor phenotypes with ER–PR– and ER+ PR–.[29] These observations suggest that tumor progression to a particular phenotype may be associated with loss of regulatory proteins.

The expression of ER variants in clinical human breast cancer has been reported.[20,31–34] Presence of tumor hormonal phenotypes characterized by ER–PR+ and ER+PR– was thought to represent a subset of breast tumors in which ER is abnormal. In tumors with ER–PR+ phenotype, one would speculate that the ER variant may have lost its steroid binding domain but remains constitutively functional with respect to induction of PR synthesis. A subset of breast cancer tumors with this phenotype has been reported.[20,32–34] Similarly, in tumors characterized with ER+PR– phenotype, it is likely that the ER, while retained its estradiol binding activity, may have lost one or more of its functional domains such as the DNA binding domain or transactivation functional domains. In such cases, one would predict that ER measurements with ligand binding assays would permit detection of ER. However, this ER may be nonfunctional. There are several reports in the literature which suggest that ER, while it binds estradiol, may fail to bind to hormone response elements and appears nonfunctional.[35] The clinical significance of these observations is that patients with ER–PR+ tumors may not respond to antiestrogen therapy due to the inability of ER variant to bind to the antiestrogen. Similarly, patients with tumors characterized by ER+PR– phenotype may not respond to antiestrogen therapy due to the nature of nonfunctional ER.

The prevalence of ER+PR– tumors did not exhibit any consistent trend with age. One explanation may be that ER+PR– tumors represent a transition phase in cell differentiation or a stage in the growth of breast cancer. These tumors may also characterize the population of breast cancer patients that do not respond to endocrine therapy due to the presence of dysfunctional ER. Since there are significant differences in the prevalence of tumor phenotype among the various age groups, it is likely that lack of ER expression or function is associated with disease progression and tumor phenotypic changes with age. We were unable to correlate these findings with other breast cancer risk factors (e.g., family history, age at menarche, parity) because such data were inaccessible.

The increased prevalence of ER+PR+ tumor phenotype with age may be attributed to upregulation of ER expression by decreased circulating levels of endocrine hormones. The decrease in the prevalence of ER–PR– and ER–PR+ tumor phenotypes with age may represent a different mechanism of regulation of

TABLE II
Median of PR, ER, and PR-to-ER Ratio in ER+PR+ Tumors by Age Group (1200 Patients)

Age group (years)	N	Median ER (fmol/mg)	Median PR (fmol/mg)	Median PR-to-ER
<45	122	34	108	3.1
45–54	159	49	98	1.7
55–64	223	87	64	0.9
65–74	334	104	83.5	0.8
>74	362	138	83	0.83

Note: Kruskal-Wallis chi-square = 129.9; $p = 0.0001$ for ER values. Kruskal-Wallis chi-square = 6.82; $p = 0.1497$ for PR values. Kruskal-Wallis chi-square = 106.3; $p = 0.0001$ for median PR-to-ER ratio.

ER expression when endocrine hormones are altered with age.[30–32] Thus, the progression of tumor phenotype from ER+PR+ to ER–PR– may be the result of tumor cell de-differentiation in response to other endocrine factors or a manifestation of tumor progression to acquire growth advantages, becoming less dependent on estrogens. Fine-needle aspirate studies[30,31] of normal and cancerous breast tissue in premenopausal women have shown that, in normal subjects, ER synthesis is cyclic and is mainly produced when plasma estrogen and progesterone concentrations are reduced (during the early follicular phase). In contrast to normal breast tissue, in older patients ER expression in breast cancer tissue may occur constitutively. Hence, among premenopausal patients, information on the menstrual-cycle stage at the time of biopsy may be important in understanding the possible effects on tumor hormonal sensitivity.[30]

The discordant tumors (ER+PR– and ER–PR+) are not clearly understood. Tumors with ER–PR+ phenotype were found mostly in the youngest age group (<45 years), and the prevalence decreased with age. This may be explained by other factors, such as the use of oral contraceptives, or by cyclic changes in plasma estradiol and progesterone levels in premenopausal women. Our findings are limited because of the lack of information regarding other possible cofounders on the association between age and ER.

The relationship of age, receptor content, and clinical outcome in breast cancer has been investigated yet remains controversial due to complexity of the factors involved. In this study, we suggest that tumor hormonal phenotypes in younger patients tend to be ER–PR+ or ER–PR– and are likely to be hormone insensitive. In contrast, tumors of older patients are more likely to be ER+PR+ and are likely to be hormone sensitive. This would indicate a higher frequency of undifferentiated tumors in younger patients and differentiated tumors in older patients. This is in agreement with observations of greater disease free survival in ER+PR+ tumors and a poor disease-free survival in ER–PR– patients. The clinical significance of ER and PR distribution with age remains under investigation.

The discrepancies in the prevalence of tumor phenotypes between our study and others may be attributed to limitations of receptor assay techniques, choice of different cutoff values for ER and PR status, or the age distribution of the population studied. Further, other factors such as tumor tissue procurement and handling, assay conditions, previous radiotherapy treatment prior to surgery, or treatment with antiestrogens prior to surgery may affect ER and PR values. Our study confirms and extends the observations made in previous reports[5,9,17,18,24] in which ER+PR+ tumors were shown to be most prevalent in older age groups. However, our study shows that ER–PR– tumors were less prevalent with age, contrary to other reports.[23] Additional prospective studies are needed to address the question of whether the four tumor phenotypes represent multiple forms of cancer or different stages in the de-differentiation process of one form of cancer.

ACKNOWLEDGMENT

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ACTIVATION OF HUMAN ESTROGEN RECEPTOR BY TEA EXTRACTS.

A.Traish, K. Murphy, L. Hafer, N. Savelyeva and A. Rogers.
Boston University School of Medicine Boston MA.

Epidemiological and experimental studies have linked intake of tea to reduced cancer risk at several sites. It has been suggested that growth of breast cancer cell lines is reduced by tea extracts. In this study, we investigated the effects of tea extracts on estrogen receptor (ER) binding and activation, as assessed by phosphorylation of ER on serine 118 in MCF-7 cells. (-) -Epigallocatechin (EGC), (-)- epigallocatechin-3-gallate (EGCG), theoflavins and green & black tea extracts displaced [^{3}H] estradiol binding from ER. The order of effectiveness was EGCG > theaflavins = green tea polyphenols = black tea polyphenols > green tea powder > black tea powder. Dixon plot analyses of the data suggested that these compounds interact with ER binding site. Treatment of MCF-7 cells for 30 min at 37^{0}C in culture with estradiol or diethylstilbestrol, but not progesterone or tamoxifen, resulted in ER phsophorylation, as did treatment with EGCG, theoflavins, green tea and black tea polyphenol extracts. These studies suggest that tea constituents possess moderate ER binding and estrogenic activity and may have physiological activity. The potential anti-carcinogenic effects of tea may be, in part, related to binding of tea polyphenols to ER and its phosphorylation. This work was supported by grants from DOD DAMD 17-94-J-4468 and DAMD 17-94-J-4421. Drs.D. Ballentine and S. Wheeler, Lipton, Inc., kindly provided the tea extracts.

TEA POLYPHENOLS BIND TO ESTROGEN RECEPTOR AND INDUCE ITS PHOSPHORYLATION. **A.M.Traish[1]*, K. Murphy[2], L. Hafer[3], N. Savelyeva[4], A.E. Rogers[5]. Departments of Biochemistry[1,2,4] & Pathology[3,5], Boston University School of Medicine, Boston, MA.**

Epidemiological and experimental studies have linked intake of tea to reduced cancer risk at several sites. It has also been suggested that growth of breast cancer cell lines is reduced by tea extracts. In this study, we investigated the effects of tea extracts on: a) estrogen receptor (ER) binding in calf uterine cytosol, and b) ER phosphorylation in MCF-7 cells. Using ligand-binding studies we determined if tea extracts bind to ER and if the binding is of a competitive nature. The data obtained show that (-) - epigallocatechin (EGC), (-) – Epigallocatechin-3-gallate (EGCG), theoflavins, and green & black tea extracts displaced radiolabeled estradiol binding from ER. The order of effectiveness was EGCG > theoflavins, = green tea polyphenols = black tea polyphenols > green tea powder > black tea powder > ECG. Dixon plot analyses of the data suggested that these compounds interact directly with ER binding site. We have developed a site-directed monoclonal antibody (EVG F9), raised against a hybrid peptide (amino acids 247-263 of ER coupled to amino acids 140-154 of ER). This antibody detects both the phosphorylated and non-phosphorylated forms of ER by immunoblotting. Since activation of ER was shown to involve phosphorylation of ER at serine 118, we determined if tea extracts activate ER, as assessed by changes in ER phosphorylation, in intact cells, at physiological temperature. Incubation of MCF-7 cells, at 37C for 30 minutes, with estradiol (1 nM), diethylstilbestrol (1 nM), EGCG (1 mg/ml), theoflavins (1 mg/ml), green tea and black tea polyphenols (1 mg/ml) resulted in ER phosphorylation. Treatment with ORG 2058 (1 nM) or tamoxifen (10 nM) did not induce receptor phosphorylation. These observations suggest that tea constituents possess moderate ER binding and estrogenic activity. The observations that tea extracts cause ER activation, as indicated by binding to ER and its phosphorylation suggest a structure/function relationship of tea polyphenols and estrogenic activity. This may explain in part the observed physiological activity of tea intake in experimental animal models. The potential anti-carcinogenic effects of tea may be related to binding of tea polyphenols to ER and their action as impeding estrogens possessing only moderate physiological activity. This study was supported by grants from DOD DAMD 17-94-J-4468 and DAMD 17-94-J-4421. We thank Drs. D. Ballentine and S. Wheeler, Lipton, Inc., for kindly providing the tea extracts.

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Loss of Expression of a 55 kDa Nuclear Protein (*nmt55*) in Estrogen Receptor-Negative Human Breast Cancer

Abdulmaged M. Traish, Ph.D., Yue-Hua Huang, Jacqueline Ashba, M.Ph., Mary Pronovost, M.D., Matthew Pavao, David B. McAneny, M.D., and Robert B. Moreland, Ph.D.

We have identified and characterized a 55 kDa nuclear protein (referred to as *nmt55*) from human breast tumors and MCF-7, human adenocarcinoma breast cell line, using site-directed monoclonal antibodies. Measurements of estrogen receptors (ER) and progesterone receptors (PR), by ligand binding assays, in cytosols of 63 human breast tumors permitted classifications of these tumors into four phenotypes (ER+/PR+, ER+/PR−, ER−/PR−, ER−/PR+). Nuclear protein (*nmt55*) expression in these tumors, as determined from Western blot analyses, showed a statistically significant association ($p = 0.001$) with tumor hormonal phenotype. Review of the pathologic characteristics of tumors analyzed suggested that lack of *nmt55* expression was significantly associated with mean tumor size ($p < 0.03$), mean ER ($p = 0.001$) and mean PR ($p < 0.002$), but was not associated with tumor stage, grade, or type. To further study this protein, we cloned and sequenced a 2.5 kb cDNA using a monoclonal antibody to *nmt55*. The complete predicted open reading frame encodes a protein with 471 amino acids and a calculated molecular mass of 54,169 Da. The deduced amino acid sequence exhibited unique regions rich in glutamine, histidine, arginine, and glutamic acid. Northern blot analysis of RNA from MCF-7 cells and ER+/PR+ human breast tumors showed a 2.6 kb mRNA. Southern blot analysis suggested the presence of a single copy of this gene. Chromosomal mapping, using fluorescent in situ hybridization (FISH), located *nmt55* gene to the X chromosome, region q13. The extensive homology between *nmt55* and RNA binding proteins suggested that *nmt55* may be involved in hnRNA splicing. The strong association observed between expression of *nmt55*, tumor hormonal phenotype, mean tumor size, mean ER, and mean PR content suggests that loss of *nmt55* expression may be related to events involved in hormone insensitivity, tumor differentiation, and unregulated tumor cell growth and metastases.

Key Words: Breast tumors—Estrogen receptor—55 kDa nuclear protein—RNA-binding protein—Splicing factors.

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The development of breast cancer is thought to be a multistage process (9). The progression of this disease is associated with cellular and molecular changes. Thus, initiation and progression may be associated with loss of chromosomal material and ultimately specific gene function(s). Some of these cellular and molecular changes may be associated with tumor cell acquisition of metastatic ability. Discovery of tumor-specific genes that lead to tumor metastasis is important in the development of strategies for treatment of breast cancer patients. There is an urgent need for identification of node-negative patients whose tumors have a metastatic potential. Several tumor markers are used in assessing tumor changes linked to poor prognosis. These include loss of estrogen receptors (ER) and progesterone receptors (PR) (18–20), high blood vessel count (angiogenesis) (13), amplification of *erbB2/HER2/neu* gene (29,33) and decreased activity of *nm23* gene (2). None of these markers alone predict, with complete reliability, which node-negative patients will be likely to relapse.

Positive and negative regulators of metastasis are likely to exist in breast cancer. During malignant progression, breast cancer cells may acquire cellular transforming functions required for abnormal growth and loss of regulatory gene functions (e.g. tumor suppressor genes). The existence of specific genes responsible for suppression of tumor metastasis has been reported (27). Over the past several years, a number of proteins were implicated in the aberrant growth of human breast cancer cells, such as epidermal growth factor receptor (EGFR) (33,41). The *erbB2* protein is under investigation for its possible use as a response variable for monitoring the efficacy of chemotherapy. Similarly, other proteins are thought to be involved in the process of metastasis, such as *p53* (a transcriptional factor with tumor suppressor properties) (41) and *nm23* (a putative metastatic suppressor) (2). While these proteins may have some prognostic value, they have yet to be utilized clinically for lack of sufficient information on these markers.

In our efforts to screen human breast tumors for ER

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From the Departments of Biochemistry (A.M.T., J.A., M.P.), Urology (Y-H.H., R.B.M.), and Surgical Oncology (M.P., D.B.McA.), Boston University School of Medicine, Boston, MA.

Address correspondence and reprint requests to Abdulmaged Traish, Ph.D., Department of Biochemistry, Boston University School of Medicine, W607, 80 East Concord St., Boston, MA 02118, U.S.A.

variants using a monoclonal antibody raised to a 15-amino acid polypeptide representing a unique sequence of the acidic/basic (A/B) region of human ER, we observed that this antibody cross-reacted with a 55 kDa nuclear protein present only in a subset of human breast cancer tissues. In this study we sought to identify and characterize this protein to assess its potential role in human breast cancer. We have identified, characterized, and cloned the cDNA for *nmt55* protein. One striking observation is that tumors that did not express ER and PR also lacked expression of *nmt55* protein. Because ER expression is used as a prognostic factor in management of breast cancer patients, it is possible that loss of *nmt55* expression may represent the state of tumor differentiation.

MATERIALS AND METHODS

Clinical Material

Human breast cancer tissue was obtained from patients undergoing surgery for treatment of breast cancer, as described previously (39). Two normal breast tissue specimens were obtained from subjects who had undergone breast reconstruction surgery. This study was approved by the Institutional Review Board of Boston University Medical Center Hospital. Human breast cancer cell line MCF-7 was obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD, U.S.A.). Monoclonal antibody NMT-1 (36,38) was developed to a unique peptide encompassing amino acids 140–154 in the A/B region of the human estrogen receptor.

Estrogen and Progesterone Receptor Assays

The concentrations of unoccupied ER and PR were determined by ligand binding analyses as described previously (35–39). Radiolabeled estradiol was used as a ligand for ER and radiolabeled ORG 2058 was used as ligand for PR (35–39). All assays were performed in The Hormone Receptor Assay Laboratory at Boston University Medical Center. The binding data were normalized in femtomoles per milligram (fmol/mg) of cytosol proteins. We have chosen cutoff values for ER as 10 fmol/mg protein and for PR as 2 fmol/mg protein. These cutoff values reflect the sensitivity of the assays using the appropriate ligands. Tumors were stratified into four groups according to their ER and PR status: 1) ER+PR+, 2) ER−PR−, 3) ER+PR−, and 4) ER−PR+. Similar tumor stratifications were reported based on ligand binding and gel shift assays (6,18–20,30).

Statistical Analysis

Statistical analyses were performed to determine the association between *nmt55* protein expression, ER and PR expression, and tumor pathologic characteristics. Two-sample *t*-test procedures were used to compare the mean values of ER and PR concentrations and tumor size with presence or absence of *nmt55*. We also conducted chi-square analyses to examine possible differences between tumor characteristics (stage, grade, tumor hormonal phenotypes, and tumor type) and *nmt55* protein status. Multiple logistic regression analyses were performed to assess the association of *nmt55* presence with ER and PR concentrations, adjusting for tumor characteristics. All statistical analyses were performed using mainframe SAS version 6.11 at Boston University.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Polyacrylamide gel electrophoresis (PAGE) and Western blot analyses were carried out as described previously (37). Briefly, cytosols and nuclear KCl extracts were prepared in the appropriate buffers, as described (35–39) and electrophoresed on 10% sodium dodecyl sulphate (SDS)/PAGE according to the method of Laemmli (16). The proteins were electrotransferred onto nitrocellulose membranes. Strips of the nitrocellulose membranes were then incubated in buffer TBST (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.5% Tween 20) containing 5% nonfat dry milk to block nonspecific binding sites for 1 h at 37°C and then probed for 2 h at 30°C with the appropriate dilution of the antibody in the same buffer containing 5% nonfat milk.

Bacterial Strains

Escherichia coli strain XL1 Blue [genotype F′::Tn10 *proAB lacI*^{q}Δ (*lacZ*) M15/*recA1 endA1 gyrA96* (Nal^r)*thi hsdR17* (r_k^- m_k+) *supE44 relA1 lac]* (Stratagene, La Jolla, CA, U.S.A.) was used for all lambda phage experiments and DH5α [genotype F′/*endA1 recA1 relA1 hsdR17* (r_k^- m_{k+}), *supE44, gyrA* (Nal^r), *thi-1*, Δ(*lacIZYA-argF*) *U169 deoR* (ϕ*80 dlac*Δ (*lacZ*)*M15*] (Life Sciences Inc., Grand Island, NY, U.S.A.) was used in the transfection and preparation of plasmid DNAs.

Library Screening and Subcloning

A lambda ZAP II (Stratagene) cDNA library of MCF-7 cells (complexity-1 × 10^7, generous gift of Dr. Mark Sobel, NCI) was screened using expression cloning with isopropylthiogalactose (IPTG)-induction (21) and Mab NMT-1 (36,38). Recombinant phage were plated at a density of ~50,000 pfu/150 mm Petri dish. Plaque lifts were prepared and probed (135 mm nitrocellulose, Schleicher and Scheull, Keene, NH, U.S.A.) as described (21). To avoid selecting ER clones, the nitrocellulose membranes were soaked in 0.1% SDS in Western blot

buffer, and then washed with buffer containing 10% methanol. This treatment denatures ER protein and does not permit detection of ER on Western blots by NMT-1. Positive plaques were isolated and purified to homogeneity. pBluescript plasmids corresponding to the positive phage were excised, according to the manufacturer's instructions. Polymerase chain reaction (PCR), using T3 and T7 RNA polymerase promoter primers (Stratagene) was carried out to determine insert sizes. The primers used flank the cloned sequences in pBluescript. Polymerase chain reaction followed by agarose gel electrophoresis revealed two clones with approximate sizes of 2.5 kb and 1.7 kb for the inserts. Restriction analysis with XhoI and EcoRI (the sites for insertion of the cDNA into lambda ZAPII) revealed a complex pattern indicating either internal EcoRI and/or XhoI restriction sites. Induction of bacteria harboring the plasmid (pBluescript *nmt55*) with 2 m*M* IPTG for 5 h followed by isolation and lysis of the bacteria in SDS sample buffer, SDS-PAGE, and Western blotting resulted in an immunoreactive ~60 kDa band, in agreement with a predicted 55 kDa protein. The 1.7 kb clone yielded a smaller protein product indicative of an internal methionine start site (data not shown).

Nucleotide Sequencing of cDNA Clones

Plasmids were prepared from candidate bacteriophage clones by cotransfection of XL-1 Blue with the helper M13 phage R408 (Stratagene) and the lambda phage. The resulting phagemids were transfected into DH5α, positive clones selected on ampicillin containing plates, screened, and plasmids were prepared. The insert DNA was sequenced in both directions using primers every 300 bp at the Boston University DNA Core Facility, using an Applied Biosystems International (ABI, Norwalk, CT, U.S.A.) 3000 DNA sequencer.

Expression of *nmt55* in Bacteria

Cultures of DH5α harboring the appropriate plasmids were grown to late log phase (A600 ~ 1A). We have observed that bacteria harboring the 2.5 kb cDNA clone did not grow when induced with IPTG, suggesting a detrimental effect of the gene product in bacteria. To overcome this, cells were grown for 3 h prior to induction with IPTG, and then induced with 2 m*M* IPTG with shaking at 300 rpm 37°C for 5 h. Aliquots of bacteria cultures were centrifuged in 1.5 ml microfuge tubes and the cell pellets lysed by boiling in 1× SDS-PAGE sample buffer. After 5 min of centrifugation to remove cell debris, aliquots of the supernatants were electrophoresed on 10% SDS-PAGE, electrotransferred, and processed for Western blot analysis.

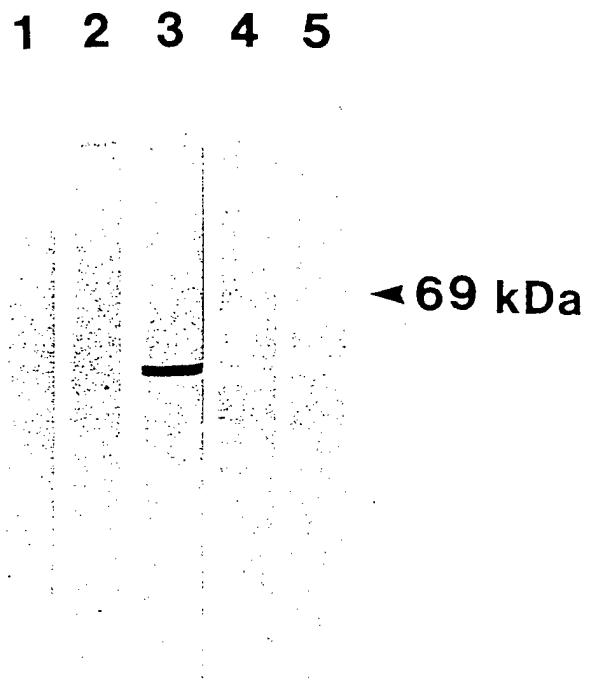


FIG. 1. Western blot analysis of MCF-7 cells nuclear and cytosolic extracts with MAb NMT-1. Nuclear KCl-extracts Lanes (lanes 1, 2, 3, and 5) and cytosol (lane 4) were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE), transferred onto nitrocellulose membranes and subjected to immunoblotting. The arrow represents the migration of bovine serum albumin (BSA, 69 kDa). Lane 1 was immunoblotted with preimmunserum; Lane 2 represents immunoabsorption of the antibody with the synthetic immunogenic peptide; Lanes 3 and 4 were immunoblotted with NMT-1 antibody; Lane 5 was immunoblotted with antibodies against RAR.

RNA Preparation and Northern Blot Analysis

Total RNA from MCF-7 cells and human breast tumor tissues was prepared by homogenization in guanidinium isothiocyanate followed by phenol/chloroform extraction and isopropanol precipitation (3). Total RNA (10–20 μg) was electrophoresed on 1% formaldehyde-MOPS agarose gels and then transferred onto nylon-reinforced nitrocellulose membranes. Membranes are treated with ultraviolet light (Stratalinker, Stratagene; 1200 watt/cm^2) to immobilize the nucleic acids. Double-stranded DNA probes for Northern blot analysis were labeled with α-[^{32}P] dCTP using T7 DNA polymerase and random primers. Specific activities range from 10^8 to 10^9 cpm/μg. Double-stranded DNA probes include a 499 bp SacI/BglII fragment of human *nmt55*, and a 545 bp HindIII/XbaI fragment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (34). The probe used for this analysis was a 499 bp SacI/BglII fragment, representing the unique carboxyl terminus of *nmt55*. This probe was chosen to avoid possible cross-hybridization with other

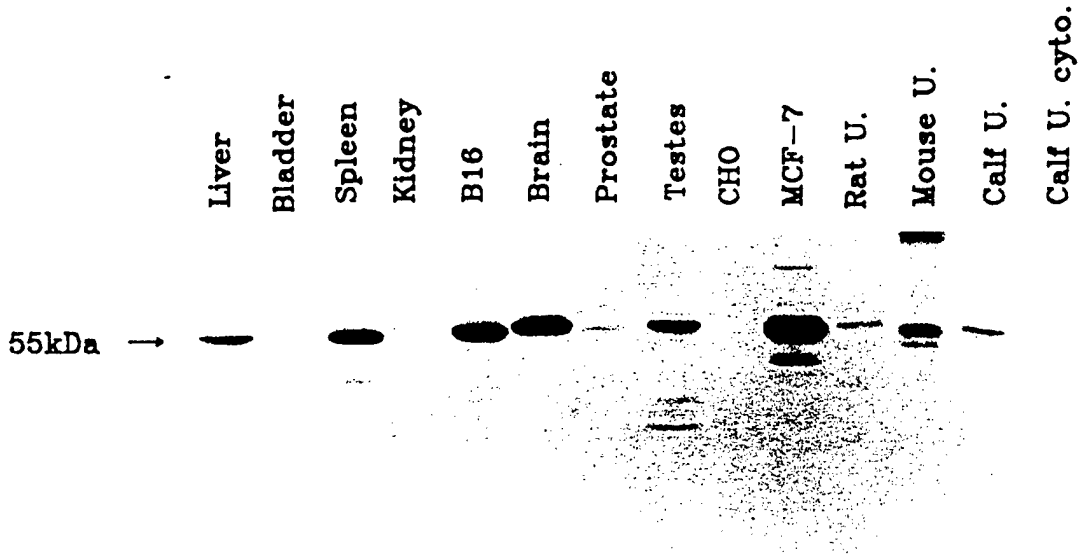


FIG. 2. Distribution of *nmt55* in tissues and cells. Rat uterine tissues were obtained from mature female animals. Mice uterine tissues were obtained from mature mice. The uteri were stripped of fat and mesenteric tissue and kept frozen at –80°C until use. Calf uterine tissues were obtained from a local slaughter house, placed on dry ice, and transported to the laboratory. Rat kidney, bladder, spleen, brain, testis, liver, and prostate were obtained from mature male animals. B16 melanoma cells were a gift from Dr. Richard Niles (Marshall University, WV). CHO cells were obtained from Dr. M. Brann (University of Vermont). Human breast cancer tissues were obtained from patients undergoing surgery for breast cancer. Tissues were placed on dry ice and transported to the laboratory where they are stored at –80°C until experimentation. Nuclear KCl-extracts from several tissues and cells were electrophoresed, electrotransferred onto nitrocellulose, and immunoblotted with MAb NMT-1.

RNA binding factors such as polypyrimidine splicing factor (PSF) (7,24). Hybridizations were carried out at 67°C for 2 h in Quickhyb (Stratagene). Following low (2× SSC 0.1% SDS, 25°C) and high stringency (0.2× SSC 0.1% SDS, 65°C) washes, membranes were exposed to Hyperfilm for 24–48 h at –70°C (Amersham, Arlington Heights, IL, U.S.A.). RNA samples were normalized for loading using GAPDH (34).

Southern Blot Analysis of Genomic DNA

Human placental genomic DNA (20 μg) was digested with restriction endonucleases (EcoRI, Hind III, NcoI, and PstI; genomic grade, high concentration), and electrophoresed on 1% agarose gels (17). The gels are transferred onto nylon-reinforced nitrocellulose membranes, and treated with ultraviolet light (Stratalinker, Stratagene; 1200 watt/cm^2) to immobilize the nucleic acids. Double-stranded DNA probes for Southern blot analysis are prepared as described in the experiments for Northern blot analysis (above), using a 499 bp Sac/BglII fragment of human *nmt55*. Hybridizations are carried out at 67°C for 2 h. Following low (2× SSC 0.1% SDS, 25°C) and high-stringency washes (0.2× SSC 0.1% SDS, 65°C), membranes are exposed to Hyperfilm for 24–48 h at –70°C.

Chromosomal Mapping

Slide Preparation

Lymphocytes from human blood were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum and phytohemagglutinin (PHA) at 37°C for 68–72 h. The lymphocyte cultures were treated with BrdU (0.18 mg/ml, Sigma) to synchronize the cell population. The synchronized cells were washed three times with serum-free medium to release the block and recultured at 37°C for 6 h in MEM with thymidine (2.5 μg/ml, Sigma, St. Louis, MO, U.S.A.). Cells were harvested and slides were made using standard procedures including hypotonic treatment, fixed and air dried.

Fluorescent In Situ Hybridization

Biotinylation of the cDNA probe (2.5 kb) with dATP using BioNick labeling kit (BRL, Bethesda Research Laboratories, Bethesda, MD, U.S.A.) (15°C, 1 h) was carried out as described (10). In situ hybridization was performed by the fluorescent in situ hybridization (FISH) method as described by Heng et al. (11,12). Slides were baked at 55°C for 1 h and after RNase treatment, DNA on the slides was denatured with 70% formamide in 2× SSC for 2 min at 70°C and dehydrated by ethanol. Probes were denatured at 75°C for 5 min in a hybridization mix

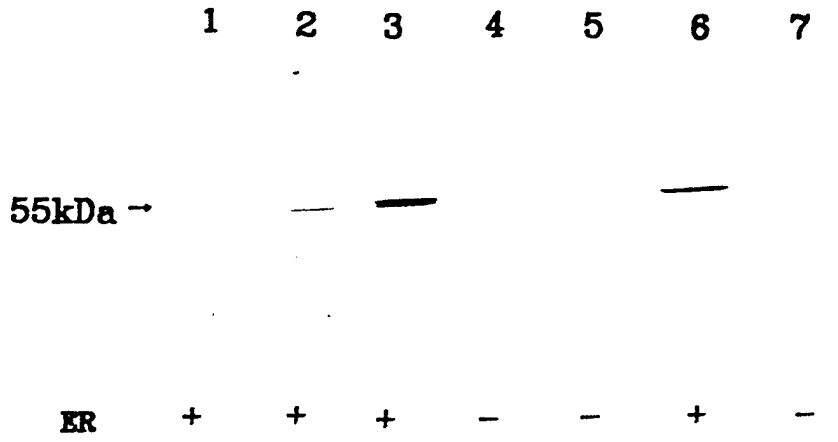


FIG. 3. Western blot analysis of *nmt55* in nuclear KCl-extracts of human breast tumors. Nuclear KCl-extracts from ER+ and ER− human breast tumors were prepared and analyzed by Western blot using NMT-1 antibody. Presence or absence of ER in these tumors is indicated at the bottom.

consisting of 50% formamide and 10% dextran sulphate. Probes were loaded onto the denatured chromosomes on slides. After overnight hybridization, slides were washed and the signal was detected and amplified. In the same field, FISH signals and DAPI banding patterns were recorded separately by fluorescent photomicroscopy using appropriate filters. The assignment of FISH mapping data with chromosomal bands was achieved by superimposing FISH signals with DAPI banded chromosomes (12).

RESULTS

Identification and Characterization of a 55 kDa Nuclear Protein in Human Breast Cancer

A monoclonal antibody (NMT-1) (36,38) raised to a unique peptide from the human ER did not recognize denatured ER on Western blots but reacted with a 55 kDa nonreceptor protein solubilized by extraction of nuclei from human MCF-7 cells with buffer containing 0.4 M KCl (Fig. 1, lane 3). The 55 kDa protein band was not detected by preimmune serum (Fig. 1, lane 1) or with antibodies to retinoic receptor (RARα) (26) (Fig. 1, lane 5), progesterone receptor (37) or with monoclonal antibodies directed to various regions of ER (1,40) (data not shown), suggesting that NMT-1 recognizes an epitope on this novel protein. Preincubation of the antibody with the peptide (amino acid 140–154 of human ER) effectively inhibited the binding of the antibody to this protein, as shown by the absence of the immunoreactivity (Fig. 1, lane 2). This protein was detected only in nuclear KCl-extracts but not in low-salt cytosolic extracts (Fig. 1, lane 4) suggesting that this protein is tightly bound to nuclear components. The inability of the monoclonal antibody NMT-1 to detect ER after SDS/PAGE was confirmed by using ER specific antibodies that detect ER in the same blots (data not shown). This suggests that NMT-1, while it detects *nmt55* protein, failed to detect ER on Western blots because its epitope on ER, once denatured by SDS, could not be renatured under these Western blot conditions.

To investigate if *nmt55* is conserved among various species and to determine its distribution in nuclear extracts from various tissues and cells, we analyzed nuclear extracts derived from various tissues and cells by Western blots. As shown in Fig. 2, *nmt55* was detected in nuclear extracts of porcine brain, rat liver, spleen, testes, and uterus. This protein was present in MCF-7 cells, and in B16 melanoma cells. This protein was not detectable in CHO cells and was detected in prostate, kidney, and bladder, albeit at a lower abundance. We have also tested if this protein is expressed in normal mammary gland. Analysis of nuclear extracts of two tissue specimens from a normal breast by Western blot demonstrated the expression of *nmt55* (data not shown). Also, immunohistochemical analysis of normal rat mammary gland showed positive staining within the epithelial and stroma, suggesting that this protein is expressed in normal mammary gland cells (data not shown). These observations suggest that this protein is conserved among species and its presence in many normal tissues indicate that it may have an important biological function.

Tumor Pathologic Characteristics

The tumors analyzed were obtained from 63 patients who underwent surgery for treatment of breast cancer. The mean age of patients was 61.5 years (SD ± 15.8) with a range of 30–95 years. The racial composition of the patients in this study was 40 (87%) white, 4 (8.7%) black, 2 (4.3%) hispanic, and 18 (28.5%) of unknown race. Fifty-two (83.9%) of 62 tumors were characterized as infiltrating ductal carcinoma, 6 (9.7%) were infiltrating lobular carcinoma, 2 (3.2%) were ductal carcinoma in situ, 1 (1.6%) lobular carcinoma in situ, and one was inflammatory. The distribution of tumors based on size was as follows: 3.4% were between 0–0.9 cm, 52.5% between 1–1.9 cm, 33.9% between 2–4.9 cm, and 10.2%

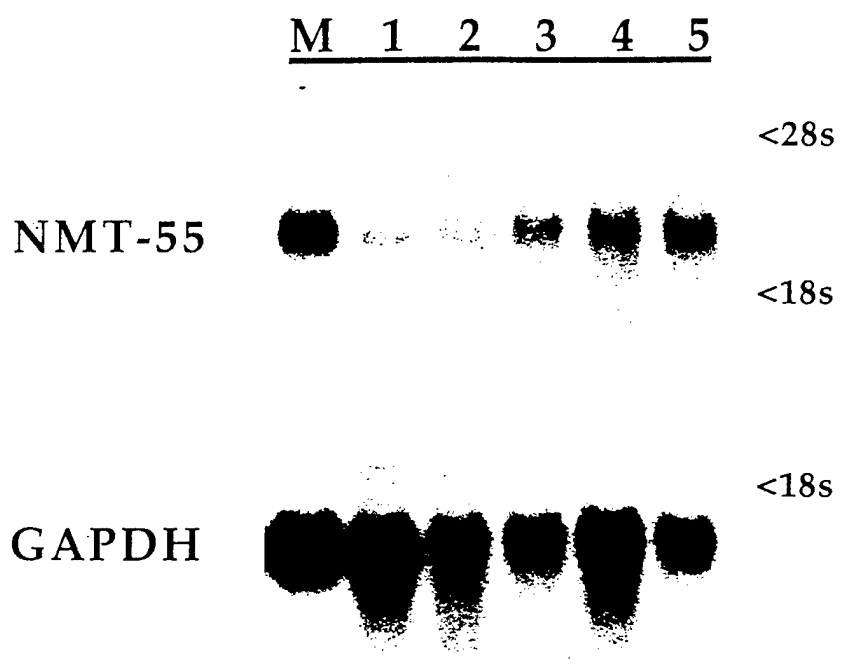


FIG. 4. Northern blot analysis of *nmt55* mRNA from MCF-7 cells and human breast tumors. Total RNA prepared from MCF-7 cells (lane M) or from human breast tumor tissue (lanes 1–5) was analyzed by northern blots as described in "Materials and Methods." GAPDH was probed to indicate levels of RNA loading.

were ⩾5 cm. Tumor stage and grade were available for 48 and 47 of the 63 patients, respectively. Approximately 44% of tumors were stage I, 33% were stage IIA or IIB and 22.9% were stage IIIA or higher. Six of 47 tumors were classified as grade 1, 24 were classified as grade 2, and 17 were classified as grade 3.

Expression of ER, PR, and *nmt55* in Human Breast Tumors

Using Western blot analysis (Fig. 3), we have analyzed the nuclear KCl-extracts of 63 human breast tumors for the presence or the absence of *nmt55*. We have also measured ER and PR content in cytosolic extracts of these tumors. We have also reviewed the pathology records of these 63 patients, as discussed above, and performed statistical analysis to determine if *nmt55* is associated with expression of biochemical markers (ER and PR) and pathologic characteristics. All tumors which expressed *nmt55* were ER+ (100%), whereas 60% of tumors lacking *nmt55* were ER+ ($p = 0.001$). Similarly, all tumors expressing *nmt55* were PR+ (100%) compared to 62.5% of tumors (PR+) in which *nmt55* was absent ($p = 0.001$). The mean tumor size was significantly greater (mean = 2.7 cm) in tumors lacking *nmt55* protein, than those in which *nmt55* was present (mean = 1.8 cm) ($p = 0.036$). The tumor hormone phenotype varied significantly by hormone status ($p = 0.001$) and all tumors expressing *nmt55* were ER+/PR+. Of tumors lacking *nmt55* 40% were ER+/PR+, 20% were ER+/PR−, 17.5% were ER−/PR−, and 22.5% were ER−/PR+.

In order to predict the absence of *nmt55* using the biochemical and pathologic data available, the logistic regression analyses was limited to 45 patients from which most of the available information was collected. The model included the following determinants: PR, ER, tumor size, and tumor stage. Tumor type was not included in the model because the majority of tumors were infiltrating ductal carcinoma (83.9%). Tumor grade was also not included due to uneven distribution in this limited sample size. PR concentration independently and inversely predicted the loss of *nmt55* protein expression, after adjusting for tumor size and tumor stage ($p = 0.014$). This implies that as PR concentration decreases the likelihood of loss of *nmt55* increases.

Because loss of ER expression and/or function correlates with poor tumor differentiation (18–20), it is possible that loss of *nmt55* expression represents poor tumor differentiation. These observations suggest that loss of *nmt55* expression may be associated with tumor dedifferentiation, loss of ER and PR expression and possibly tumor metastases (18–20). Thus, understanding of the role of this protein in breast cell growth and function requires detailed studies to determine its biological and biochemical function. As a first step towards this goal, we have pursued cDNA cloning and characterization of *nmt55* gene in human breast cancer cells.

Cloning of *nmt55* From MCF-7 cDNA Library

Two clones were isolated and sequenced; one represented a full-length 2.5 kb clone and a 1.7 kb clone that was an internal fragment of the 2.5 kb. The 2.5 kb cDNA encompassed 115 bp 5′ untranslated, a 1,416 bp open

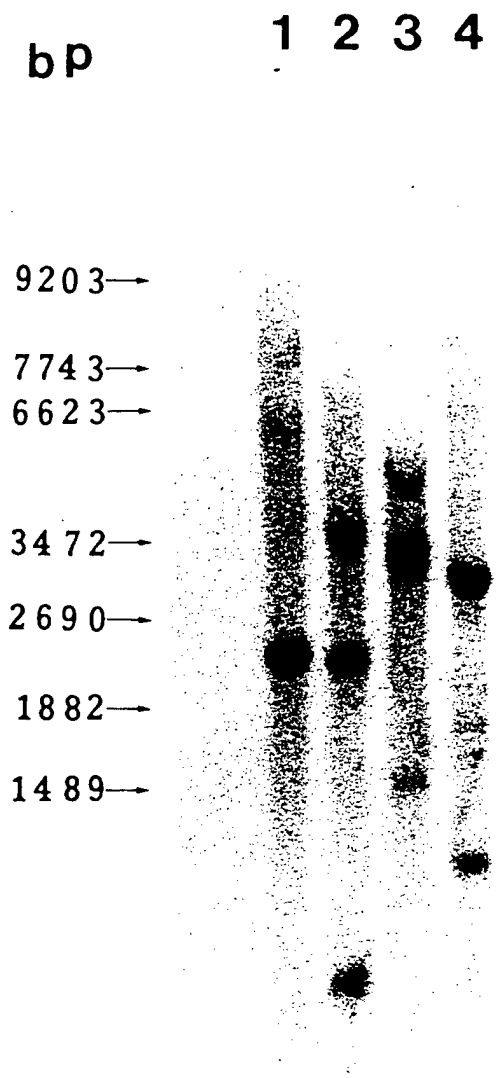


FIG. 5. Southern blot analysis of *nmt55* genomic DNA. Genomic DNA isolated from human placenta was digested with EcoRI (lane 1), HindIII (lane 2), PstI (lane 3), and NcoI (lane 4) and electrophoresed on 1% agarose gels. The DNA was transferred onto nylon-reinforced nitrocellulose and hybridized with the radiolabeled probe (a 499 bp SacI/BglII cDNA fragment). Subsequent to high-stringency washes the blot was dried and exposed to X-ray film. The size of the radiolabeled bands was determined by molecular weight markers (Lambda DNA digested with StyI).

reading frame, and ~970 bp of 3′ untranslated sequence terminating in poly A. The open reading frame was predicted to encode a 471 amino acid protein (54169 Da), in good agreement with the observed 55 kDa apparent molecular weight determined by Western blots from nuclear KCl extracts. No unique sequences were identified with homology to ER, except 5 amino acids (Ala Ala Pro Gly Ala) which were found in the C-terminal region of *nmt55* and represent the epitope of NMT-1 (see below).

Characterization of *nmt55* mRNA Expression by Northern Blot Analysis

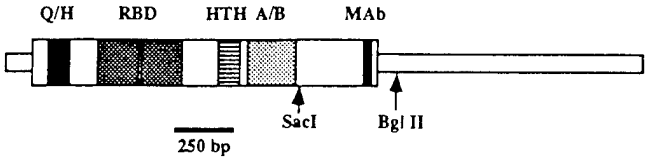
Figure 4 (lane M) shows that MCF-7 cells express a relatively abundant 2.6 kb mRNA transcript for *nmt55*. Analysis of total RNA from five ER+/PR+ human breast tumors (lanes 1–5) demonstrated different levels of nmt-55 mRNA expression. The low levels of expression observed in tumors represented by lanes 1 and 2 are not due to different RNA loading, since GAPDH mRNA levels were similar for all tumor samples. In preliminary experiments, tumors that were ER– and PR– did not express detectable *nmt55* transcripts (data not shown). The tumors used in these experiments also expressed different levels of *nmt55* protein, as determined by Western blot analysis (data not shown).

Southern Blot Analysis

Human female placental genomic DNA was digested with EcoRI (Fig. 5, lane 1), HindIII (lane 2), PstI (lane 3) and NcoI (lane 4) and probed with a 499 bp SacI/BglII cDNA fragment. The product of EcoRI digestion hybridized to a single 2.3 kb band, suggesting the presence of a single copy of this gene. Deoxyribonucleic acid digested with HindIII showed three bands (3.5, 2.3, and 0.7 kb) despite the presence of a single HindIII site in the cDNA approximately 330 bp 3′ to the probe. This suggests the presence of one or more intervening sequences in this region. This observation is further supported by the hybridization products subsequent to digestion with PstI (~4.4, 3.2, and 1.4 kb), since PstI site is absent in the cDNA. Digestion with NcoI resulted in hybridization to a 2.9 and 1.1 kb bands. Since the two NcoI sites predicted from the cDNA sequence flank the probe and would be expected to yield only a 0.96 kb fragment, the hybridization pattern obtained suggests that the 2.9 and 1.1 kb bands must be the result of intervening sequences.

Structural Features of *nmt55* Protein: Amino Acid Sequences, Location of the RNA Binding Domains and Antibodies Binding Epitopes

The amino acid sequence of *nmt55*, deduced from the cDNA, is shown in Fig. 6. Several interesting features are noted: glutamine- and histidine-rich regions (Q/H) located in the amino terminus (residues 19–35); a predicted bipartite RNA binding domain (RBD), residues 75–147 and 149–228; a putative helix-turn-helix motif (HTH), residues 287–335; and a region rich in acidic

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86/1                                                    116/11
ATG CAG AGT AAT AAA ACT TTT AAC TTG GAG AAG CAA AAC CAT ACT CCA AGA AAG CAT CAT
 M   Q   S   N   K   T   F   N   L   E   K   Q   N   H   T   P   R   K   H   H
146/21                                                  176/31
CAA CAT CAC CAC CAG CAG CAG CAC CAC CAG CAG CAA CAG CAG CAG CCG CCA CCA CCG CCA
 Q   H   H   H   Q   Q   Q   H   H   Q   Q   Q   Q   Q   Q   P   P   P   P   P
206/41                                                  236/51                  NMT4
ATA CCT GCA AAT GGG CAA CAG GCC AGC AGC CAA AAT GAA GGC TTG ACT ATT GAC CTG AAG
 I   P   A   N   G   Q   Q   A   S   S   Q   N   E   G   L   T   I   D   L   K
266/61                                                  296/71
AAT TTT AGA AAA CCA GGA GAG AAG ACC TTC ACC CAA CGA AGC CGT CTT TTT GTG GGA AAT
 N   F   R   K   P   G   E   K   T   F   T   Q   R   S   R   L   F   V   G   N
326/81                                                  356/91
CTT CCT CCC GAC ATC ACT GAG GAA GAA ATG AGG AAA CTA TTT GAG AAA TAT GGA AAG GCA
 L   P   P   D   I   T   E   E   E   M   R   K   L   F   E   K   Y   G   K   A
386/101                                                 416/111
GGC GAA GTC TTC ATT CAT AAG GAT AAA GGA TTT GGC TTT ATC CGC TTG GAA ACC CGA ACC
 G   E   V   F   I   H   K   D   K   G   F   G   F   I   R   L   E   T   R   T
446/121                                                 476/131
CTA GCG GAG ATT GCC AAA GTG GAG CTG GAC AAT ATG CCA CTC CGT GGA AAG CAG CTG CGT
 L   A   E   I   A   K   V   E   L   D   N   M   P   L   R   G   K   Q   L   R
506/141                                                 536/151
GTG CGC TTT GCC TGC CAT AGT GCA TCC CTT ACA GTT CGA AAC CTT CCT CAG TAT GTG TCC
 V   R   F   A   C   H   S   A   S   L   T   V   R   N   L   P   Q   Y   V   S
566/161                                                 596/171
AAC GAA CTG CTG GAA GAA GCC TTT TCT GTG TTT GGC CAG GTA GAG AGG GCT GTA GTC ATT
 N   E   L   L   E   E   A   F   S   V   F   G   Q   V   E   R   A   V   V   I
626/181                                                 656/191
GTG GAT GAT CGA GGA AGG CCC TCA GGA AAA GGC ATT GTT GAG TTC TCA GGG AAG CCA GCT
 V   D   D   R   G   R   P   S   G   K   G   I   V   E   F   S   G   K   P   A
686/201                                                 716/211
GCT CGG AAA GCT CTG GAC AGA TGC AGT GAA GGC TCC TTC CTG CTA ACC ACA TTT CCT CGT
 A   R   K   A   L   D   R   C   S   E   G   S   F   L   L   T   T   F   P   R
746/221                                                 776/231
CCT GTG ACT GTG GAG CCC ATG GAC CAG TTA GAT GAT GAA GAG GGA CTT CCA GAG AAG CTG
 P   V   T   V   E   P   M   D   Q   L   D   D   E   E   G   L   P   E   K   L
806/241                                                 836/251
GTT ATA AAA AAC CAG CAA TTT CAC AAG GAA CGA GAG CAG CCA CCC AGA TTT GCA CAG CCT
 V   I   K   N   Q   Q   F   H   K   E   R   E   Q   P   P   R   F   A   Q   P
866/261                                                 896/271
GGC TCC TTT GAG TAT GAA TAT GCC ATG CGC TGG AAG GCA CTC ATT GAG ATG GAG AAG CAG
 G   S   F   E   Y   E   Y   A   M   R   W   K   A   L   I   E   M   E   K   Q
926/281                                                 956/291
CAG CAG GAC CAA GTG GAC CGC AAC ATC AAG GAG GCT CGT GAG AAG CTG GAG ATG GAG ATG
 Q   Q   D   Q   V   D   R   N   I   K   E   A   R   E   K   L   E   M   E   M
986/301                                                 1016/311
GAA GCT GCA CGC CAT GAG CAC CAG GTC ATG CTA ATG AGA CAG GAT TTG ATG AGG CGC CAA
 E   A   A   R   H   E   H   Q   V   M   L   M   R   Q   D   L   M   R   R   Q
1046/321                                                1076/331
GAA GAA CTT CGG AGG ATG GAA GAG CTG CAC AAC CAA GAG GTG CAA AAA CGA AAG CAA CTG
 E   E   L   R   R   M   E   E   L   H   N   Q   E   V   Q   K   R   K   Q   L
1106/341                                                1136/351
GAG CTC AGG CAG GAG GAA GAG CGC AGG CGC CGT GAA GAA GAG ATG CGG CGG CAG CAA GAA
 E   L   R   Q   E   E   E   R   R   R   R   E   E   E   M   R   R   Q   Q   E
1166/361                                                1196/371            NMT5
GAA ATG ATG CGG CGA CAG CAG GAA GGA TTC AAG GGA ACC TTC CCT GAT GCG AGA GAG CAG
 E   M   M   R   R   Q   Q   E   G   F   K   G   T   F   P   D   A   R   E   Q
1226/381                                                1256/391
GAG ATT CGG ATG GGT CAG ATG GCT ATG GGA GGT GCT ATG GGC ATA AAC AAC AGA GGT GCC
 E   I   R   M   G   Q   M   A   M   G   G   A   M   G   I   N   N   R   G   A
1286/401                                                1316/411
ATG CCC CCT GCT CCT GTG CCA GCT GGT ACC CCA GCT CCT CCA GGA CCT GCC ACT ATG ATG
 M   P   P   A   P   V   P   A   G   T   P   A   P   P   G   P   A   T   M   M
1346/421                                                1376/431
CCG GAT GGA ACT TTG GGA TTG ACC CCA CCA ACA ACT GAA CGC TTT GGT CAG GCT GCT ACA
 P   D   G   T   L   G   L   T   P   P   T   T   E   R   F   G   Q   A   A   T
1406/441                                                1436/451            NMT1
ATG GAA GGA ATT GGG GCA ATT GGT GGA ACT CCT CCT GCA TTC AAC CGT GCA GCT CCT GGA
 M   E   G   I   G   A   I   G   G   T   P   P   A   F   N   R   A   A   P   G
1466/461                                                1496/471
GCT GAA TTT GCC CCA AAC AAA CGT CGC CGA TAC TAA
 A   E   F   A   P   N   K   R   R   R   Y   *
```

FIG. 6. Schematic representation of *nmt55* cDNA and the predicted amino acid sequence. The locations of the glutamine- and histidine-rich region (Q/H), the RNA binding domain (RBD), the helix-turn-helix mofit (HTH), the acidic/basic residues region (A/B) are indicated. The 499 bp SacI/BglII fragment used as a probe for Northern and Southern blots is also shown. The peptide sequences used to develop antibodies were underlined.

(A)

RNA Binding Domain

```
nmt55          75 RLFVGNLPPD ITEEEMRKLF EKYGKAGEVF IHKDKGFGFI RLETRTLAEI
HeLa p54nrb    75 RLFVGNLPPD ITEEEMRKLF EKYGKAGEVF IHKDKGFGFI RLETRTLAEI
Mouse NonO     77 RLFVGNLPPD ITEEEMRKLF EKYGKAGEVF IHKDKGFGFI RLETRTLAEI
PSF           298RLFVGNLPAD ITEDEFKRLF AKYGEPGEVF INKGKGFGFI KLESRALAEI
NonA/BJ6      303RLYVGNLTND ITDDELREMF KPYGEISEIF SNLDKNFTFL KVDYHPNAEK

nmt55         125AKVELDNMPL RGKQLRVRFA CHSASLTVRN LPQYVSNELL EEAFSVFGQV
HeLa p54nrb   125AKVELDNMPL RGKQLRVRFA CHSASLHVRN LPQYVSNELL EEAFSVFGQV
Mouse NonO    127VKVELDNMPL RGKQLRVRFA CHSASLTVRN LPQYVSNELL EEAFSVFGQV
PSF           348AKAELDDTPM RGRQLRVRFA THAAALSVRN LSPYVSNELL EEAFSQFGPI
NonA/BJ6      353AKRALDGSMR KGRQLRVRFA PNATILRVSN LTPFVSNELL YKSFEIFGPI

nmt55         175ERAVVIVDDR GRPSGKGIVE FSGKPAARKA LDRCSEGSFL LTTFPRPVTV
HeLa p54nrb   175ERAVVIVDDR GRPSGKGIVE FSGKPAARKA LDRCSEGSFL LTTFPRPVTV
Mouse NonO    177ERAVVIVDDR GRPSGKGIVE FSGKPAARKA LDRCSEGSFL LTTFPRPVTV
PSF           398ERAVVIVDDR GRSTGKGIVE FASKPAARKA FERCSEGVFL LTTTPRPVIV
NonA/BJ6      403ERASITVDDR GKHMGEGIVE FAKKSSASAC LRMCNEKCFF LTASLRPCLV

nmt55         225EPMD
HeLa p54nrb   225EPMD
Mouse NonO    227EPMD
PSF           448EPLE
NonA/BJ6      453EPME
```

(B)

Helix Turn Helix Motif

```
nmt55          287 RNIKEAREKL EMEMEAARHE HQVMLMRQDL MRRQEELRRM EELHNQEVQ
HeLa p54nrb    287 RNIKEAREKL EMEMEAARHE HQVMLMRQDL MRRQEELRRM EELHNQEVQ
Mouse NonO     289 RNIKEAREKL EMEMEAARHE HQVMLMRQDL MRRQEELRRM EELHNQEVQ
```

FIG. 7A. Homologies between *nmt55* RNA recognition motif and other related proteins.
FIG. 7B. Homologies between *nmt55* helix-turn-helix motif and other related proteins.

(glutamic) and basic (arginine) residues (A/B) extending from residue 318 to residue 368. These structural features, together with its nuclear localization, suggest that this protein may play a role as a nucleic acid binding protein (42).

When the open reading frame was screened against the GenBank database, two clones of high homology were found, one murine (42) and one human (4). One of these clones isolated from HeLa cells has high homology to PSF family of RNA splicing factors and contains a bipartite RNA binding motif (23,24). The murine candidate had high homology to the OCT-2 family of transcription factors as well as RNA binding motifs. As shown in Fig. 7A, the RNA recognition motifs of *nmt55* share considerable homologies with those of HeLa p54^{nrb} (4) PSF (24), *NonO* (42) and NonA/BJ6 (28). Also, *nmt55* has a homologous region in the predicted helix-turn-helix motif with HeLa p54^{nrb}, and *NonO* (Fig. 7B).

Using the deduced amino acid sequence, we have developed two new polyclonal antibodies to *nmt55*: one antibody (NMT-4) raised to a peptide in the N-terminal region that encompasses amino acids 56–72 of *nmt55* and another antibody (NMT-5) raised to a peptide in the carboxyl terminal region and spans amino acids 371–386 of *nmt55*. The peptides were chosen to avoid cross-reactivity of the generated antibodies with other RNA binding proteins that share homology with *nmt55* (e.g. PSF). Western blot analysis showed that only one band was detected with these antibodies when nuclear extracts from MCF-7 cells and human breast tumors were tested (data not shown). These antibodies should be useful in developing enzyme-linked immunosorbent assay (ELISA) sandwich assays for routine and quantitative detection of this protein in human breast tumor nuclear extracts. Immunocytochemical analysis of rat mammary gland tissues showed only nuclear localization in both epithelial and stromal cells (data not shown).

Chromosomal Mapping with the FISH Method

Seventy-four of 100 mitotic figures showed signal on one pair of the X chromosome (Fig. 8, panels A and B). Since DAPI was used to identify the specific chro-

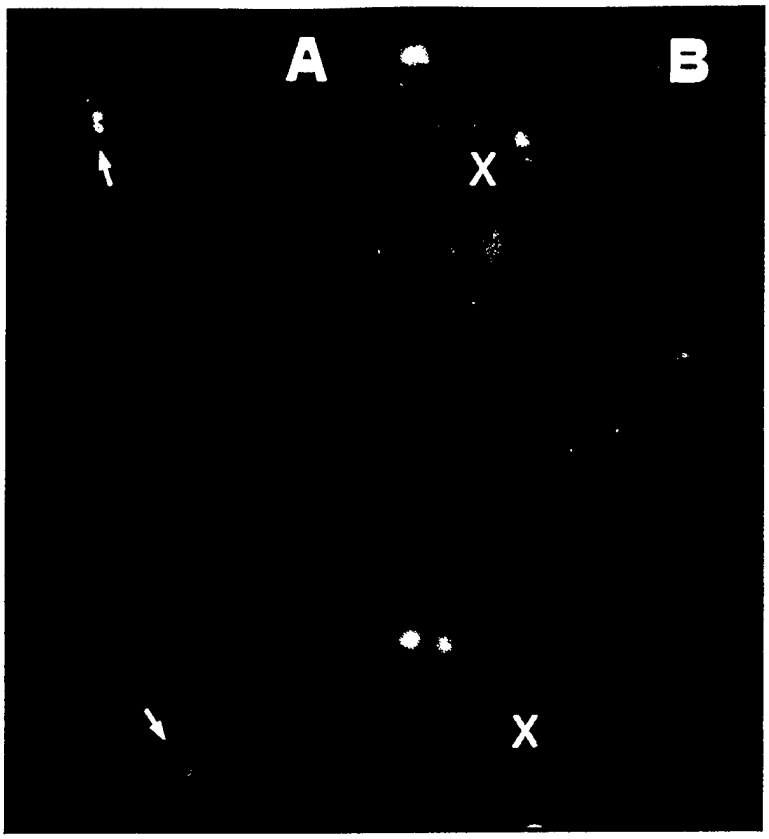

FIG. 8. Chromosomal location of *nmt55* gene. Fluorescent in situ hybridization (FISH) mapping of nmt55 gene showed signals on chromosome X (Panel **A**). Mitotic figures stained with DAPI are shown in Panel **B**.

mosomes, the assignment between signal from the cDNA probe and the long arm of chromosome X was obtained. The detailed position of the *nmt55* gene was further assessed from the summary analyses of 10 photographs, and was assigned to the long arm of chromosome X q13, as depicted in Fig. 9. We did not detect additional loci for *nmt55* using the FISH method.

DISCUSSION

In this study we have identified and characterized a 55 kDa protein (*nmt55*) using a site-directed monoclonal antibody to a unique peptide derived from human ER. Although NMT-1 monoclonal antibody was raised against a peptide from ER, NMT-1 failed to react with denatured ER in Western blot analyses. This observation suggests that the epitope for this antibody on ER is very sensitive to conformational changes, and once denatured by SDS it could not be recognized in ER. In contrast, this epitope appears to be stable or capable of renaturation in *nmt55*, as demonstrated by consistent detection by Western blot analyses. Nuclear protein *nmt55* did not bind estradiol, and did not cross-react with antibodies specific to ER (1,40). These observations suggested that *nmt55* was not an ER variant. It also did not associate with ER, as assessed by sucrose density gradient analysis and subsequent Western blotting. In addition, *nmt55* was localized mainly to the nucleus and is found in many tissues of animal species. This suggests that *nmt55* is conserved and may have an important role in cellular regulation. Nuclear protein *nmt55* expression was detected in normal breast tissue biopsies and in most ER+/PR+ breast tumors, but not expressed in all of ER– human breast tumors. Loss of ER expression in human breast tumors is often associated with poor prognosis, while continued expression of ER is associated with disease-free survival and correlates well with tumor state of differentiation (18–20). It is possible that the loss of *nmt55* expression in the majority of ER– human breast tumors is related to loss of regulation of normal cellular growth and function.

Evaluation of tumor pathologic characteristics and hormonal status and the relationship with *nmt55* expression showed an association with ER status, PR status, tumor hormone phenotype, and mean tumor size. The association of tumor size with loss of *nmt55* expression suggests that a possible role for this protein in tumor growth, invasion, or metastases. Since it has been proposed that tumor size is an indicator of tumor metastases (15), it is possible that loss of *nmt55* expression in large tumors may be indicative of tumor progression to metastases. This would raise the possibility that loss of *nmt55* may be a potential marker for tumor metastases. The PR was an independent predictor of *nmt55* protein expression. Because PR is under estrogen control, it is possible that the observed relationship between tumor hormonal status and *nmt55* expression is related to es-

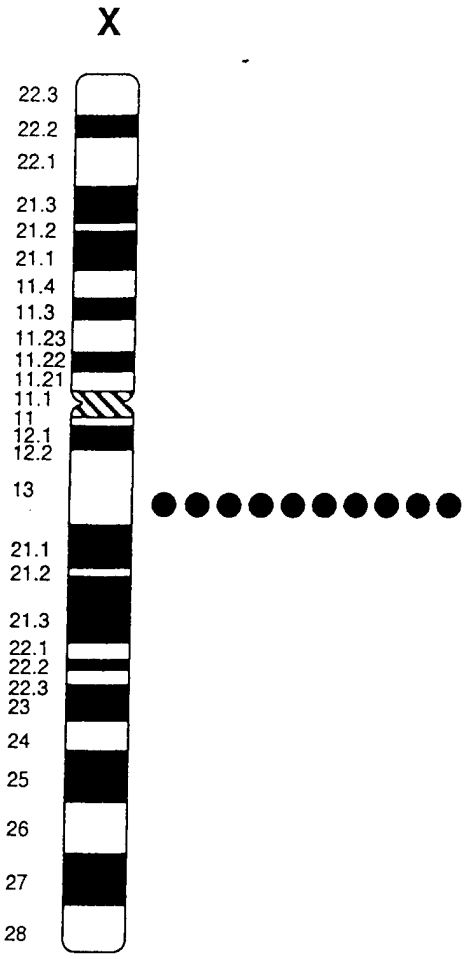

FIG. 9. Diagram of fluorescent in situ hybridization (FISH) chromosomal location of *nmt55* gene. Each dot represents the double FISH signals detected on human chromosome X.

trogen receptor function. Tumors that continue to express ER but not PR (ER+/PR−) may express nonfunctional ER and that these tumors are progressing to a poor state of differentiation (6,18–20,30).

We have cloned the cDNA for *nmt55* from a human breast tumor cell line. The predicted open reading frame encodes a 471 amino acid protein with a calculated molecular weight of 54,169 Da. The deduced amino acid sequence of this protein indicated that it is a basic protein with an estimated pI of 9.5. Based on homology with other proteins, *nmt55* has two RNA recognition motifs (RRM), suggesting that it is an RNA binding protein. The presence of a region with helix-turn-helix structure in *nmt55* suggests that *nmt55* binds to DNA. While the structural features are suggestive of RNA binding proteins (14), further work is necessary to demonstrate that *nmt55* indeed binds specifically to RNA.

A homology search of the GenBank/EMBL databases identified two other related genes. One was described by Dong et al., (4) cloned from HeLa cells and another is a mouse *NonO* (42). Nuclear protein *nmt55* had amino acid sequence identity >97 and 99% with *NonO* (42) and HeLa $p54^{nrb}$ (4), respectively. Nuclear protein *nmt55* differed from HeLa $p54^{nrb}$ in one amino acid; in *nmt55*, amino acid 151 was Thr while in HeLa $p54^{nrb}$ amino acid 151 is His. The mRNA for *nmt55* and HeLa $p54^{nrb}$ (4) detected by northern blot analysis revealed a similar size mRNA (~2.6 kb). Comparison of Southern blot analysis of *nmt55* and HeLa $p54^{nrb}$ (4), however, revealed subtle differences. While EcoRI digest of genomic DNA revealed a single 2.3 kb with an *nmt55* cDNA probe, EcoRI digest of genomic DNA probed by a homologous $p54^{nrb}$ cDNA probe showed >8.5 kb, 6 kb and 2.3 kb bands (4). When digested with PstI, *nmt55* showed three bands (~4.4, 3.2, and 1.4 kb) while $p54^{nrb}$ showed only two bands (~4.9 and 3.3 kb). Fluorescent in situ hybridization analyses of *nmt55* localized the gene to only one chromosomal locus on Xq13. The high degree of sequence homology and the detection of only one locus for *nmt55* by FISH suggest that *nmt55* and $p54^{nrb}$ are either the same gene or closely related genes tandemly arranged on Xq13.

Although the role of *nmt55* in cellular function is unknown at present, the striking homology between *nmt55* and HeLa $p54^{nrb}$ (4), 60 kDa murine *NonO* (42), and PSF (24) suggest that *nmt55* may be a member of a family of splicing factors. These proteins exhibit conserved bipartite RNA binding domains common to proteins implicated in RNA splicing (4,24,42). HeLa $p54rnb^{nrb}$ was cloned using antibodies to yeast splicing factor PRP18 and has high homology to PSF and the Drosophila puff protein BJ6-$nonA^{diss}$ (28). *NonO* is a 60 kDa murine homologue of Drosophila $nonA^{diss}$ which has a bipartite RNA binding motif, and an OCT-2-like helix-turn-helix lacking the POU domain (42). PSF is a 100 kDa protein shown to be essential for RNA splicing, but lacks the transcription factor-like domains and interacts with a 60 kDa RNA binding protein PTB (8). While *nmt55* and PTB have similar molecular weights, they share little amino acid homologies (8). Nuclear protein, hnRNP-A2/B1, which belongs to a family of RNA processing proteins was shown to be a marker for detection of lung cancer (43). It is therefore, possible that proteins involved in pre-mRNA processing may be important in development or progression of neoplasm.

Several studies have attempted to elucidate the function of RNA binding proteins (e.g. PSF, PTB, U2AF, Drosophila Sex Lethal [*SxL*]) (14,32). These proteins recognize distinct polypyrimidine tracts in the 3′ splice sites of hnRNAs. These proteins are thought to regulate alternative splicing by selectively repressing 3′ splice sites containing a binding site for their respective RNA binding proteins (32). It has been suggested that proteins that contain RRM share a common fold in a similar protein-RNA interface and may participate in pre-

mRNA processing via a common mechanism of function (14).

In summary, loss of *nmt55* expression may be an early marker for loss of ER expression or function. The strong association between *nmt55* and PR expression clearly indicates that *nmt55* may be an important marker in breast cancer. The ER gene encompasses more than 140 kb and is subdivided into 8 exons yielding a 6.32 kb mRNA (25). It is possible that *nmt55* is involved in pre-mRNA processing of ER, and loss of its expression in tumor cells may result in loss of ER expression due to lack of proper mRNA splicing. This may explain tumor heterogeneity, especially with respect to hormonal interventions in the course of treatment and further illustrates the heterogeneity of this disease. Also, since loss of *nmt55* expression is associated with increased tumor size, it is possible that this gene may be involved in tumor metastases. Therefore, *nmt55* may represent a new marker of tumor progression and prognostic information. While the function of *nmt55* remains unknown at present, its potential role as a splicing factor may be critical to cell growth and function and its loss of expression in human breast tumors may indicate loss of normal growth. The exact function of this protein and the significance of its gene location on the X chromosome q13 near the centromere are currently under investigation. □

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The Inhibitory Effects of Transforming Growth Factor-β1 on Breast Cancer Cell Proliferation are Mediated Through Regulation of Aberrant NF-κB/Rel Expression

Mika A. Sovak[1,3], Marcello Arsura[2,3], Gregory Zanieski[2,3], Kathryn T. Kavanagh[1,3], and Gail E. Sonenshein[2,3*]

Departments of Pathology and Laboratory Medicine[1] and Biochemistry[2], and Program in Research on Women's Health[3], Boston University Medical School, 715 Albany Street, Boston MA 02118

Running title: Growth inhibition by TGF-β1 mediated via NF-κB/Rel

*Corresponding author, Tel: (617) 638-4120; FAX: (617) 638-5339;

E-mail: gsonensh@bu.edu

NF-κB/Rel transcription factors normally exist in non-B cells, such as epithelial cells, in inactive forms sequestered in the cytoplasm with specific inhibitory proteins, termed IκBs. Recently, however, we discovered that breast cancer is typified by aberrant constitutive expression of NF-κB/Rel factors. Since these factors control genes that regulate cell proliferation, here we analyzed the potential role of NF-κB/Rel in the ability of TGF-β1 to inhibit growth of breast cancer cells. Decreased growth of Hs578T and MCF7 breast cancer cell lines upon TGF-β1 treatment correlated with a drop in NF-κB/Rel binding. This decrease was due to stabilization of the inhibitory protein IκB-α. Ectopic expression of c-Rel in Hs578T cells led to maintenance of NF-κB/Rel binding and resistance to TGF-β1-mediated inhibition of proliferation. Similarly, expression of the p65 subunit ablated the inhibition of Hs578T cell growth mediated by TGF-β1. Thus, inhibition of the aberrantly activated, constitutive NF-κB/Rel plays an important role in arrest of proliferation of breast cancer cells, suggesting NF-κB/Rel may be a useful target in the treatment of breast cancer.

Transforming growth factor-β1 (TGF-β1) belongs to a family of polypeptides that plays a role in cellular proliferation, development, and extracellular matrix modeling. TGF-β1 has been demonstrated to have significant inhibitory effects upon the growth of numerous cell types (1-3), including mammary epithelial cells. *In vitro* studies have revealed its inhibitory effects upon the proliferation of numerous primary human and established breast epithelial cell lines (4,5). Evidence for a role of TGF-β1 in normal mammary gland development and proliferation was provided by studies in mice. Transgenic mice expressing TGF-β1 linked to the mouse mammary tumor virus (MMTV) promoter displayed high levels of TGF-β1 in the mammary gland (6). These mice were demonstrated to have hypoplastic mammary duct development (6). In addition, Silberstein et al. (7) demonstrated that the temporary placement of slow-release TGF-β1 pellets in the mammary glands of virgin mice reversibly inhibited both mammary ductal growth and DNA synthesis.

The growth inhibitory actions of TGF-β1 have also been demonstrated in breast tumor cell lines (4,8-10). This effect has been observed in both estrogen-receptor (ER) positive (MCF7) and ER negative cells (Hs578T) (8), although some studies have suggested that ER negative cells, which are often found in more advanced breast cancers, are less susceptible to the effects of TGF-β1 (rev. in 11). The effects of TGF-β1 on breast cancer have also been seen *in vivo*. For example, the MMTV-TGF-β1 transgenic mice displayed increased resistance to the breast carcinogenic effects of 7,12-dimethylbenzanthracene (DMBA) (6). Furthermore, when these mice were crossed with MMTV-transforming growth factor alpha (TGF-α) transgenic mice, which show increased incidence of both spontaneous and DMBA-induced breast tumors, the offspring displayed decreased incidence of both spontaneous breast tumors as well as resistance

to the tumorigenic effects of DMBA compared to the parental TGF-α transgenics (6). These studies suggest that TGF-β1 significantly inhibits the development of breast cancer.

There are several theories as to the mechanism of action of TGF-β1. Studies have linked its growth inhibitory effects to the down-regulation of genes involved in cellular proliferation, such as cyclin-dependent kinases (12-14), the retinoblastoma susceptibility product (pRB) (15,16) and the c-*myc* proto-oncogene (16,17). Recent results from our lab have shown that the TGF-β1 treatment of immature B cells and hepatocytes involves a novel signaling mechanism exerted through down-regulation of the Nuclear Factor-kappaB (NF-κB)/Rel family of transcription factors (18,19). NF-κB/Rel is a family of dimeric transcription factors whose members all contain a 300 amino acid Rel homology domain (RHD) (20,21). In mammalian cells, subunit members include p50 or NF-κB1, p52 or NF-κB2, p65 or RelA, c-Rel, and RelB (20). NF-κB/Rel factors are involved in control of genes implicated in regulation of cellular proliferation, cell survival, adhesion, immune and inflammatory responses (20,21). The activity of NF-κB/Rel factors is controlled post-translationally by their subcellular localization. In most cells, other than mature B lymphocytes, NF-κB/Rel proteins are sequestered as inactive forms in the cytoplasm by association with inhibitory proteins, termed IκB's, for which IκB-α represents the prototype (22-25). Activation involves IκB phosphorylation, resulting in its ubiquitination and subsequent degradation, which then allows for nuclear translocation of the NF-κB/Rel protein (25). Recently we reported that NF-κB/Rel is aberrantly activated in human breast cancer and in rat mammary tumors induced by DMBA (26). Thus, here we have investigated the role of NF-κB/Rel in TGF-β1-mediated inhibition of Hs578T and MCF-7 breast cancer cell proliferation. We report that inhibition of cell growth upon TGF-β1 treatment of these tumor cell lines correlates with a decrease in NF-κB/Rel activity due to increased stability of IκB-α protein.

Furthermore, ectopic expression of c-Rel or p65 in Hs578T cells ablates the TGF-β1-mediated decrease in NF-κB/Rel activity and inhibition of growth. These finding provide evidence for a direct role of NF-κB/Rel in the TGF-β1-mediated decrease in proliferation of breast cancer cells.

RESULTS

TGF-β1 Decreases Breast Tumor Cell Line Proliferation

To confirm the effects of TGF-β1 treatment on the proliferation of Hs578T and MCF7 breast cancer cells, the effects of this cytokine on cell numbers were monitored. Concentrations of TGF-β1 ranging from 1 to 5 ng/ml have been reported to effectively inhibit breast cancer cell proliferation (4,8,27,28). Exponentially growing Hs578T and MCF7 cells were therefore treated for 72 hours with either TGF-β1 dissolved in 4% BSA carrier solution or carrier solution alone as control. Cell numbers were then determined. As can be seen in Fig. 1, TGF-β1 treatment for 3 days resulted in fewer MCF7 and Hs578T cells compared to controls. Similarly, percent labeled nuclei values were largely reduced in Hs578T or MCF7 cells that have been treated with TGF-β1 for 72 hrs compared to BSA-treated control cells (data not shown). The effects of TGF-β1 were likely due to inhibition of cellular proliferation as opposed to induction of cell death, since visual analysis of nuclear morphology did not reveal a significant number of apoptotic cells (data not shown). Thus, as seen previously (4,5), TGF-β1 potently inhibits growth of breast cancer cell lines.

TGF-β1 Decreases NF-κB/Rel Expression

TGF-β1 has been demonstrated to exert its inhibitory effects on proliferation through numerous mechanisms, including through the downregulation of NF-κB/Rel activity (18,19). To determine whether TGF-β1 affects the aberrant NF-κB/Rel activity in breast cancer cells, EMSA analysis was performed. Nuclear extracts were prepared from Hs578T and MCF7 cells incubated in the presence of 1ng/ml TGF-β1 or carrier BSA for 72 hours. The upstream NF-κB element from the c-*myc* gene (URE) was used as probe (29). As shown in Fig. 2A, TGF-β1 treatment decreased the total levels of NF-κB/Rel binding activity in both cell lines. To determine the kinetics of the TGF-β1-mediated downmodulation of NF-κB/Rel activity, Hs578T cells were treated for 24 or 48 hours with TGF-β1 and analyzed by EMSA. As shown in Fig. 2B, TGF-β1 treatment caused a significant decrease of NF-κB/Rel binding after 24 hr, that was followed by an even more pronounced downmodulation at the 48 hr time point. These effects were specific in that TGF-β1 treatment did not alter Octomer-1 (Oct-1) binding (Fig. 2C). Thus, TGF-β1 decreases both breast tumor cell proliferation and NF-κB/Rel binding activity.

TGF-β1 Increases the Half-Life of IκB-α Protein

In hepatocyte cell lines, the decrease in NF-κB activity in response to TGF-β1 treatment was mediated through an increase in IκB-α protein specifically; the levels of IκB-β, the other major IκB protein, were unaffected (19). Thus, the effects of TGF-β1 treatment of Hs578T cells on the rate of turnover of IκB proteins were assessed. Hs578T cells were incubated for 48 hrs in the presence of TGF-β1 or BSA carrier solution as control, and then treated with the protein synthesis inhibitor emetine for 1, 2 or 4 hrs. Cytoplasmic extracts were then subjected to

immunoblot analysis for the two predominant IκB proteins, IκB-α and IκB-β (Fig. 3A). A significant decrease in IκB-α degradation was noted upon TGF-β1 treatment. Densitometry was performed on the resulting immunoblots and the relative levels plotted as a function of time in Figure 3B. In the BSA-treated cells, IκB-α protein had a half life of decay of approximately 2.75 hrs (Fig. 3B). TGF-β1-treatment increased the normal half life beyond 4 hrs. In contrast, IκB-β protein had a longer half-life and no change was detected over the 4 hr time course (Fig. 3A). TGF-β1 treatment of MCF-7 cells similarly increased the half-life of IκB-α compared to BSA-carrier treated control cells, i.e., a 12 hr half-life was determined following 48 hrs TGF-β1 treatment compared to the normal 5.3 hrs in control cells. Thus, TGF-β1 treatment stabilizes IκB-α protein, lengthening the normal rate of decay.

Ectopic c-Rel Expression Ablates the Inhibitory Effects of TGF-β1 on Hs578T Cells.

To test whether ectopic expression of a transactivating NF-κB/Rel subunit was sufficient to rescue breast cancer cells from TGF-β1-induced cell growth arrest, populations of Hs578T cells expressing ectopic c-Rel were prepared. To this purpose, the murine c-Rel expression vector pSV-SPORT-c-Rel, which encodes a full-length c-Rel protein, was chosen. Hs578T cells were then stably transfected with the pSV-SPORT-c-Rel vector and the neomycin resistance construct pSV$_2$*neo* DNA, and selected for G418 resistance, as described in Materials and Methods. Two individual clonal lines were then isolated from the mixed population of resistant cells by limiting dilution. While studies with individual clones usually give more substantial effects, use of mixed populations confirms that an observation is not specific to only a few individual cells within a population. The mixed population c-Rel transfected Hs578T cells (Hs578TR) were first tested for the extent of cell growth inhibition following TGF-β1 treatment, as compared to the response

of the parental Hs578T cell line, using an MTS conversion assay. Treatment of Hs578T cells, plated at either 20% or 40% confluence, with TGF-β1 for 24 or 48 hrs resulted in a significant decrease in cell proliferation (Fig. 4A). In contrast, TGF-β1 treatment had significantly more modest effects on cell proliferation of Hs578TR cells (Fig. 4A).The Hs578TR cells were next characterized for the effects of TGF-β1 treatment on NF-κB/Rel binding activity (Fig. 4B). As expected, NF-κB/Rel binding was dramatically diminished following TGF-β1 treatment of the parental Hs578T cells for 24 and 48 hrs. The nuclear extracts from untreated Hs578TR cells displayed a higher level of NF-κB/Rel binding activity compared to those from the parental Hs578T cells (Fig. 4B). Furthermore, the nuclear extracts from Hs578TR cells retained significantly higher levels of NF-κB/Rel binding activity even after TGF-β1 treatment. These studies demonstrate that ectopic expression of a member of the NF-κB/Rel family prevents the drop in NF-κB/Rel and rescues cells from the growth inhibition mediated by TGF-β1.

The clones were next monitored specifically for c-Rel expression by immunoblotting. Cultures of individual clones of the Hs578TR and parental Hs578T cells were compared for expression of c-Rel (Fig. 5A). The parental Hs578T cells contained extremely low levels of c-Rel, in agreement with our previous report (26). All of the individual clones expressed c-Rel at levels significantly higher than the parental Hs578T cells. Several of the clones (Hs578TR-C1, -C2, -C5) expressed high levels of c-Rel, whereas Hs578TR-C3 and -C4 expressed moderately increased levels compared to the parental line. Specificity of the c-Rel protein detected was confirmed by competition with the cognate peptide (Fig. 5B). The effects of TGF-β1 on growth were further explored using the two individual c-Rel expressing clones Hs578TR-C1 and -C2. The effects of TGF-β1 treatment were monitored in two ways, MTS assay and percent labeled nuclei. TGF-β1 mediated inhibition of cell proliferation was almost completely ablated in

cultures plated at 20% confluence (Fig. 5C), or at 70% confluence (data not shown). Similarly, the TGF-β1 mediated inhibition of DNA synthesis was significantly ablated by expression of c-Rel (Fig. 5D). Thus, maintenance of higher levels of NF-κB/Rel activity protects cells from TGF-β1-induced growth arrest.

Ectopic Expression of p65 Ablates the Growth Inhibitory Effects of TGF-β1. To determine whether expression of the p65 subunit would similarly alter the response of Hs578T cells to TGF-β1, these cells were transiently transfected using FUGENE reagent, which permits a much higher efficiency of transfection. Cells were transfected either with a human p65 pMT2T expression vector or with the parental pMT2T vector DNA. A green fluorescent protein (GFP) expression vector was added to estimate transfection efficiency, which was estimated at approximately 70% based on GFP staining. Following a 48 hr treatment with TGF-β1, cell proliferation was assessed by MTS assay, and the average of two experiments, carried out in triplicate, are shown in Fig. 6. The reduction of HS578T cell proliferation normally seen upon TGF-β1 treatment was greatly ablated upon expression of p65. These results, taken together with the findings presented above, indicate that expression of either RelA or c-Rel can significantly reduce the growth-inhibitory activity of TGF-β1 on Hs578T breast cancer cells.

DISCUSSION

Here we provide evidence that the drop in NF-κB/Rel plays an important role in TGF-β1-mediated inhibition of breast cancer cell growth. TGF-β1 treatment of Hs578T and MCF7

human breast cancer cell lines decreased their rate of proliferation, and concomitantly decreased the overall levels of NF-κB/Rel binding activity in these cells. Ectopic expression of either c-Rel or p65 led to resistance to the growth inhibitory effects of TGF-β1, demonstrating a direct role of NF-κB/Rel factors in control of proliferation. Stabilization of IκB-α specifically was implicated in the observed decrease in NF-κB/Rel activity. Overall, these studies indicate that NF-κB/Rel activity is important in the control of breast tumor cell proliferation. Previously, activation or constitutive NF-κB/Rel expression has been reported to promote growth of various cell types, including B and T lymphocytes, fibroblasts, smooth muscle and liver cells (20,21, 30-35). Our results extend the growth promoting role of NF-κB/Rel factors to neoplastically transformed breast epithelial cells; furthermore, they suggest that targeting this activity may be useful in the treatment of breast cancer.

The inhibitory effects of TGF-β1 on breast cancer have been demonstrated *in vivo* with transgenic mouse studies. MMTV-TGF-β1 transgenic mice were highly resistant to DMBA-induced tumorigenesis, suggesting that overexpression of TGF-β1 has profound inhibitory effects upon breast cancer development (6). The resistance of the MMTV-TGF-β1 mice to DMBA suggests that TGF-β1 counteracts the actions of DMBA. It is possible that developmental effects of TGF-β1 expression decrease the mammary gland susceptibility to DMBA-induced tumorigenesis. However, the glands in these animals were still able to differentiate normally during pregnancy, indicating that development of the gland was not severely impaired. Interestingly, we have recently shown that mammary tumors induced in female Sprague-Dawley rats treated with DMBA are typified by aberrant induction of NF-κB/Rel activity (26). Specifically we observed that over 85% of tumors expressed high levels of nuclear NF-κB/Rel. Based on the work presented here, it is tempting to speculate that inhibition

of NF-κB/Rel activity might play an important role in the ability of TGF-β1 to interfere with the DMBA-induced tumorigenic process.

Zugmaier and coworkers (8) studied the effects of this TGF-β1 on numerous breast cancer cell lines and were able to inhibit the growth of most cell lines independent of estrogen receptor status. However, there was a discrepancy in the response of MCF7 cells depending on passage number. Early passage (<100) MCF7 cells were inhibited by TGF-β1, while late passage (>500) cells were resistant to its effects (data not shown), suggesting that biological changes that occur with continuous passage *in vitro* are in part responsible for the variable phenotype of this cell line. The MCF7 cells used in these experiments were above passage number 154 (as provided by the ATCC), and were still sensitive to the growth inhibitory effects of this agent.

While there are published reports describing MCF7 cells undergoing apoptosis after treatment with TGF-β1 (10, 36), several other studies also did not observe apoptosis of breast cancer cells upon TGF-β1 treatment (8). In our experiments, TGF-β1 did not appear to induce apoptosis in either MCF7 or Hs578T cells, as judged by propidium iodide staining of chromatin, DNA laddering or TUNEL assay. In our previous study, apoptosis was induced in 40% of Hs578T cells microinjected with IκB-α (26). There are several explanations for the apparent lack of cell death in the current studies. It is possible that partial inhibition of this factor slows cellular growth, and that complete and rapid inhibition is necessary to induce apoptosis. Interestingly, residual NF-κB/Rel binding levels was observed in both cell types after TGF-β1 treatment. Also similar to differential sensitivity to growth inhibitors (8), sensitivity to the apoptotic effects of TGF-β1 may change with passage number. Alternatively, cells may be differentially sensitive to apoptotic stimuli depending on the stage of the cell cycle.

The studies presented here indicate that inhibition of constitutive NF-κB/Rel activity may not be sufficient to induce apoptosis in all breast cancer cells, however, downregulating this activity may decrease proliferation of breast tumor cells. Several studies have found an association between response to TGF-β1 and poor prognosis in patients (36-40). The findings raised the question of whether loss of responsiveness to TGF-β1 is associated with progression to more aggressive breast tumors. Our findings raise the intriguing possibility that resistance to TGF-β1, which is most often due to the loss of TGF-β1 receptor (27, 41- 43), can be circumvented since the same inhibitory effects on tumor cell proliferation may be achieved via use of inhibitors of NF-κB. Thus, inhibition of NF-κB may provide a new method to sensitize breast cancer cells to chemotherapeutic treatments.

MATERIALS AND METHODS

Cell lines culture conditions and treatments. MCF7 cells were kindly supplied by F. Foss (Boston University Medical School, Boston, MA) and C. Sonnenschein (Tufts University Medical School, Boston, MA), or purchased from the ATCC. Human breast cancer Hs578T cells were kindly supplied by M. Sobel (NCI, Bethesda, MD), or purchased from the ATCC. MCF7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Gaithersburg, Maryland), 100 ug/ml streptomycin (Gibco), and 100 U/ml penicillin (Gibco). Hs578T cells were propagated in DMEM with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 4.5 g/L glucose, 10 ug/ml of insulin (Sigma), and antibiotics as above. Cells were plated the day before treatment: Hs578T cells at densities ranging from 2,600 - 4,300 cells/cm^2 and MCF7 cells at 7,100 - 14,000 cells/cm^2 depending on

length of treatment. A sterile stock solution of TGF-β1 (R & D Systems, Minneapolis, MN) was made to 5 ng/μl dissolved in 0.1% carrier BSA solution. The final concentration of TGF-β1 in the media culture was 1-5 ng/ml as indicated; controls were treated with equal amounts of BSA carrier solution.

For analysis of cells entering DNA synthesis, percent of cells incorporating [^{3}H]-thymidine was measured. Briefly, cells were labeled for 6 hours with 2 uCi/ml, fixed, and percent labeled nuclei assessed, as we have described previously (44). For the Non-radioactive Cell Proliferation Assay (Promega), cells were seeded at the indicated confluence in 96 well tissue culture dishes. TGF-β1- and BSA-treated cultures were incubated, in triplicate, for 4-6 hrs in the presence of MTS tetrazolium salt compound (MTS) solution (333 ug/ml) and 25 uM phenazine methosulfate according to the manufacturer's directions. The A490 was measured using an ELISA plate reader. For studies on the half-life of IκB-α, cells were plated the day before to achieve 50% confluency on the day of treatment (Hs578T cells, 2 x 10^5/p100; MCF7, 5.2 x 10^5/p60). A stock solution of 20 mg/ml of emetine (Sigma) was made in water, and cells treated with 20 ug/ml for various lengths of time as indicated.

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared from breast cancer cells by a modification of the method of Dignam et al. (45). Cells were washed twice with ice cold PBS (Ca^{2+} and Mg^{2+} free) containing protease inhibitors (0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10 ug/ml leupeptin (LP)). They were then resuspended in 1 ml of cold hypotonic RSB buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris pH 7.4) containing 0.5% NP-40 detergent plus protease inhibitors as above. Following a 15 minute incubation on ice, the cells were dounce homogenized until cell lysis occurred. Nuclei were resuspended in 2 packed nuclear volumes of extraction buffer C (420 mM KCl, 20 mM HEPES

pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol) plus protease inhibitors as above, and incubated on ice for 30 min. Protein concentration was determined using the Bio-Rad protein assay, following the manufacturer's directions. For labeling of the NF-κB URE or Oct-1 oligonucleotides, a 150-300 ng sample was incubated for 30 min at 37°C in a solution adjusted to a final concentration of 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20 uM each of dATP and dTTP, 50 uCi each of [^{32}P]-dCTP and [^{32}P]-dGTP, and 5 units of Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA). The URE oligonucleotide, described previously (29), has the following sequence: 5'-GATCCAAGTCCGGGTTTTCCCCAA CC-3'. The underlined sequences indicate the core binding elements. The Oct-1 oligonucleotide has the following sequence: 5'-TGTCGAATGCAAATCACTAGAA-3'. For the binding reaction, ^{32}P-labeled oligonucleotide (20,000 - 25,000 cpm) was incubated with 5 ug of nuclear extract, 5 ul sample buffer (10 mM HEPES, 4 mM DTT, 0.5% Triton X-100, and 2.5% glycerol), 2.5 ug poly dI-dC as nonspecific competitor, and the salt concentration adjusted to 100 mM using buffer C. The reaction was carried out at room temperature for 30 min. DNA/protein complexes were subjected to electrophoresis at 11 V/cm and resolved on a 4.5% polyacrylamide gel (using 30% acrylamide/0.8 % bisacrylamide) with 0.5x TBE running buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA pH 8.0).

Immunoblot analysis. For cytoplasmic and nuclear extracts, cells were washed twice with PBS containing DTT, PMSF and LP, as above described, and resuspended in 200-400 µl lysis buffer (10 mM Tris pH 7.6, 10 mM KCl, 5 mM $MgCl_2$) containing the above protease inhibitors, and 1% NP-40, and incubated in ice for 5 min. Nuclei were pelleted for 4 min at 2,500 rpm at 4°C. The supernatant containing cytoplasmic proteins was stored at -80°C. The nuclear pellet was

washed once in lysis buffer without detergent, centrifuged, and the nuclear proteins extracted using 100-300 ul RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% sodium lauryl sarcosine, 1% NP-40, 0.1% SDS, 1 mM EDTA) plus DTT, PMSF and LP. The DNA was sheared by pulling the solution 20 times first through a 23G and then a 25G7/8 needle. Following microcentrifugation for 30 min at 14,000 g at 4°C, the supernatant containing the nuclear proteins was removed and stored at -80°C. Protein concentrations were determined using the Bio-Rad *Dc* protein assay. Proteins samples (20-40 μg) were resolved in a 10% polyacrylamide-SDS gel, transferred to PVDF membrane (Millipore, Bedford, MA), and subjected to immunoblotting, as previously described (26). The antibodies preparation for IκB-α (SC-371), IκB-β (SC-945), and c-Rel (SC-070) were purchased from Santa Cruz Biotechnology Inc.

Isolation of Hs578T Stable Transfectants. Activity of the murine c-Rel expression vector pSV-SPORT-c-Rel (kindly provided by T. Gilmore, Boston University, Boston MA), which encodes a full-length c-Rel protein, was confirmed by transient transfection analysis in 3T3 cells, which do not express constitutive NF-κB/Rel factors (47), (D.W. Kim and G.E.S., unpublished observations). The Hs578T c-Rel stable transfectants were prepared using 38 ug pSV-SPORT-c-Rel and 2 ug pSV2*neo* DNA. Cells were transfected by calcium phosphate as described (26). After 24 hrs, 1.2 mg/ml G418 (Gibco) was added to the medium and selective growth conditions maintained for approximately 2 weeks. Clones were isolated by limiting dilution.

Transient Transfection of Hs579T Cells. Hs578T cells were plated, in triplicate, at 70% confluence in 96 well dishes. After removal of the media, cells were incubated for 24 hrs in a 4 ul solution of DNA in FUGENE Transfection reagent (Boerhinger/Mannheim), according to the manufacturer's directions. DNA used per well was either 130 ng of human p65 pMT2T (kindly

provided by U. Siebenlist, NIH, Bethesda MD) or parental pMT2T DNA, plus 20 ng GFP expression plasmid (kindly provided by C. Gelinas, Robert Wood Johnson Medical School, Piscataway NJ). After 24 hrs, the cells were treated either with carrier BSA or with 5 ng/ml TGF-β1, and the effects on growth were measured by MTS assay after 48 hrs. An approximate 70% transfection efficiency was estimated using GFP staining.

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Figure Legends

Figure 1. TGF-β1 treatment decreases proliferation of breast tumor cell lines. MCF7 and Hs578T (578T) breast tumor cell lines were plated in duplicate and treated with 1 ng/ml TGF-β1or carrier BSA solution as control. Cell numbers were determined after 3 days of treatment.

Figure 2. TGF-β1 treatment decreases nuclear NF-κB/Rel binding activity in breast tumor cell lines. A) NF-κB binding. MCF7 and Hs578T (578T) breast tumor cell lines were plated at 1.1 x 10^4 cells/Pl00 or 5.6 x 10^4 cells/P100, respectively, and treated for 3 days with 1 ng/ml TGF-β1 or carrier BSA solution as control. Nuclear extracts were made and subjected to EMSA using as probe the URE NF-κB oligonucleotide. B) NF-κB binding. Hs578T breast tumor cell lines were treated with carrier BSA for 48 hrs (C) or with 1 ng/ml TGF-β1 for 24 or 48 hrs, as indicated. Nuclear extracts were prepared and processed as in part A. Asterisk (*) indicates a nonspecific band that did not change with TGF-β1 treatment. C) Oct-1 binding. Hs578T breast tumor cell lines were treated with 1 ng/ml TGF-β1 for 0, 24 or 48 hrs. Nuclear extracts were prepared and subjected to EMSA for Oct-1 binding.

Figure 3. TGF-β1 treatment increases the half-life of decay of IκB-α in Hs578T breast cancer cells. A) Cultures of Hs578T cells were plated at a density of 2 x 10^5 cells/P100 dish the day prior to treatment. Cells were treated with 5 ng/ml TGF-β1 or carrier solution for 48 hours, and then incubated in the presence of the protein synthesis inhibitor 20 ug/ml emetine for 0, 1, 2, or 4 hrs. Cytoplasmic extracts were prepared and equal amounts (30 ug) subjected to

immunoblot analysis for IκB-α or IκB-β. B) The autoradiograph for a representative experiment for IκB-α was quantified by densitometric analysis and the data presented as a function of the amount present at time zero, set at 100% for the control and TGF-β1-treated samples. Open circles, BSA control; Closed circles, treated with TGF-β1.

Figure 4. Ectopic c-Rel expression in the Hs578TR cell population. Cultures of Hs578T cells in exponential growth were transfected with 22.5 ug of the murine c-Rel expression vector pSPORT-c-Rel and 2.5 ug pSV2Neo DNA. G418-resistant stable transfectants were isolated. A) Parental Hs578T and Hs578TR cells were plated at 20% and 40% confluence and treated with 2 ng/ml TGF-β1 or BSA as control for 24 and 48 hrs. The effects of TGF-β1 on growth were measured by MTS assay. Cell numbers are for TGF-β1-treated cells are given as percent values relative to BSA-treated control cells. B) Transfected Hs578TR (578TR) and parental Hs578T (578T) cells were plated at 40% confluence and treated with 2 ng/ml TGF-β1 for 24 and 48 hrs. Nuclear extracts were isolated and subjected to EMSA using the URE NF-κB oligonucleotide as probe. Asterisk (*) indicates a nonspecific band that did not change with TGF-β1 treatment.

Figure 5. Ectopic c-Rel expression in individual clones ablates TGF-β1-mediated growth inhibition. Individual clones (C1-C5) were isolated from the Hs578TR mixed population by limiting dilution. A and B) Immunoblot analysis. Expression of c-Rel in the individual clones was determined by immunoblot analysis. Nuclear extracts were isolated from exponentially growing clones and from parental Hs578T cells and equal samples (80 ug) were subjected to immunoblot analysis using an anti-c-Rel antibody (SC070, Santa Cruz Biotechnology) in the absence (A) or presence (B) of a 1:1 molar ratio of cognate peptide. Asterisk (*) indicates

position of a nonspecific band. C) MTS Proliferation assay. Parental Hs578T and clonal Hs578TR-C1 and Hs578TR-C2 cells were plated at 20% confluence and treated with 5 ng/ml TGF-β1 or BSA as control for 24 and 48 hrs. The effects of TGF-β1 on growth were measured by MTS assay. Cell numbers are for TGF-β1-treated cells are given as percent values relative to BSA-treated control cells. D) DNA synthesis. Parental Hs578T and clonal Hs578TR-C1 and Hs578TR-C2 cells were treated, in duplicate, with 5 ng/ml TGF-β1 for 48 hrs or BSA as control. Cells were then incubated in media containing 2 uCi of [^{3}H]-thymidine per ml for 6 hours, fixed and exposed for autoradiography. Percent labeled nuclei was determined by visual counting. Mean and standard deviation were determined in two different experiments. Black bars, parental Hs578T cells; grey bars, clone 1; white bars, clone 2.

Figure 6. Ectopic expression of p65 ablates TGF-β1 mediated growth arrest of Hs578T cells. Hs578T cells were plated, in triplicate, at 70% confluence in 96 well dishes. After removal of the media, cells were incubated according to the manufacturer's directions for 24 hrs in a 4 ul solution of DNA in FUGENE [either pMT2T parental + 20 ng GFP/well, or 130 ng of human p65 pMT2T + 20 ng GFP/well (+p65)]. After 24 hrs, the cells were treated with carrier BSA (B) or with 5 ng/ml TGF-β1 (T) and the effects of TGF-β1 on growth measured by MTS assay. The average of two experiments are shown; values are given as percent cell proliferation relative to BSA carrier-treated control cells that had been transfected with the parental pMT2T vector DNA. Transfection efficiency was estimated to be approximately 70% based on GFP staining.

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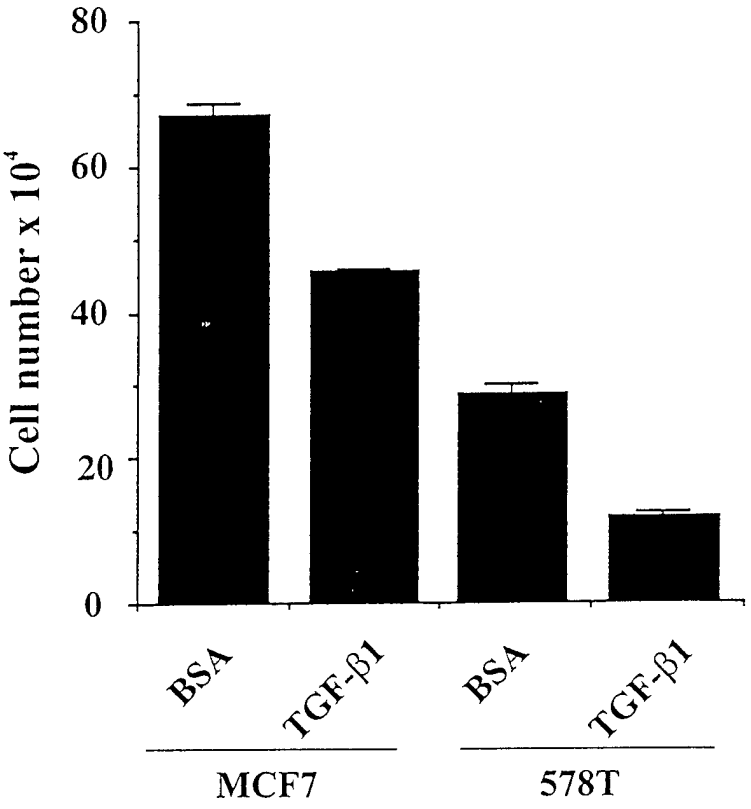


Figure 1

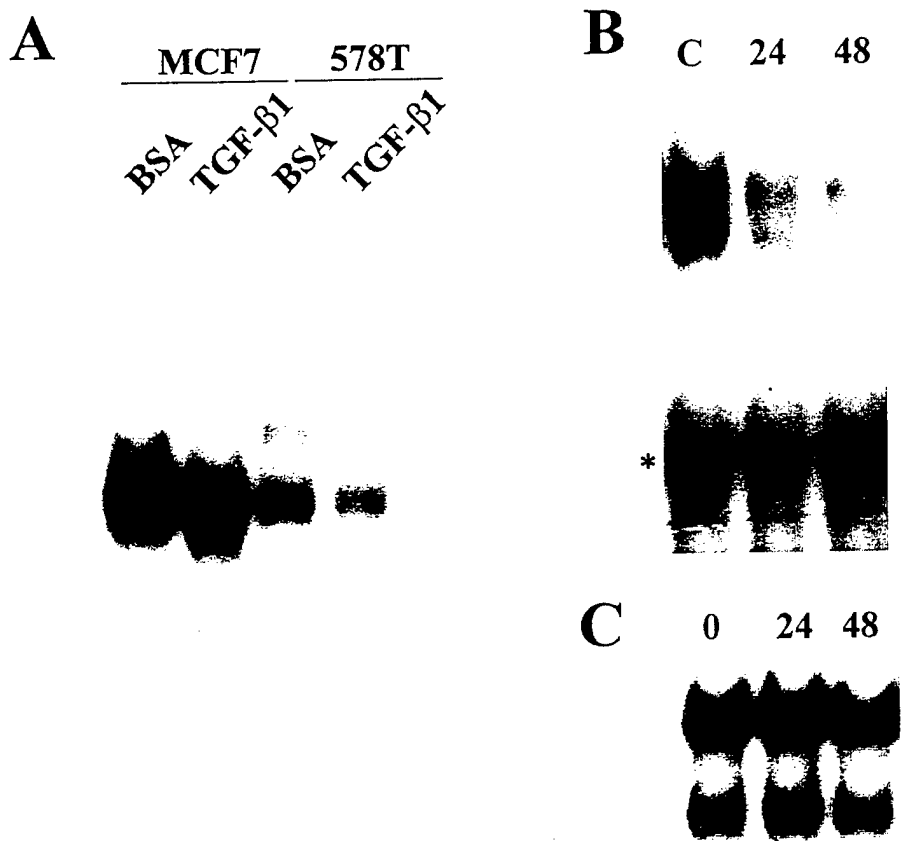


Figure 2

A

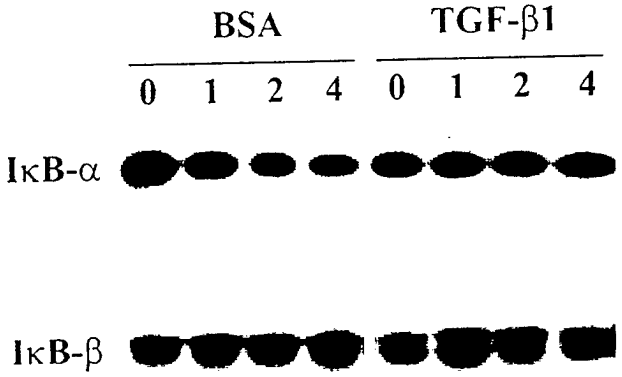


Figure 3A

B

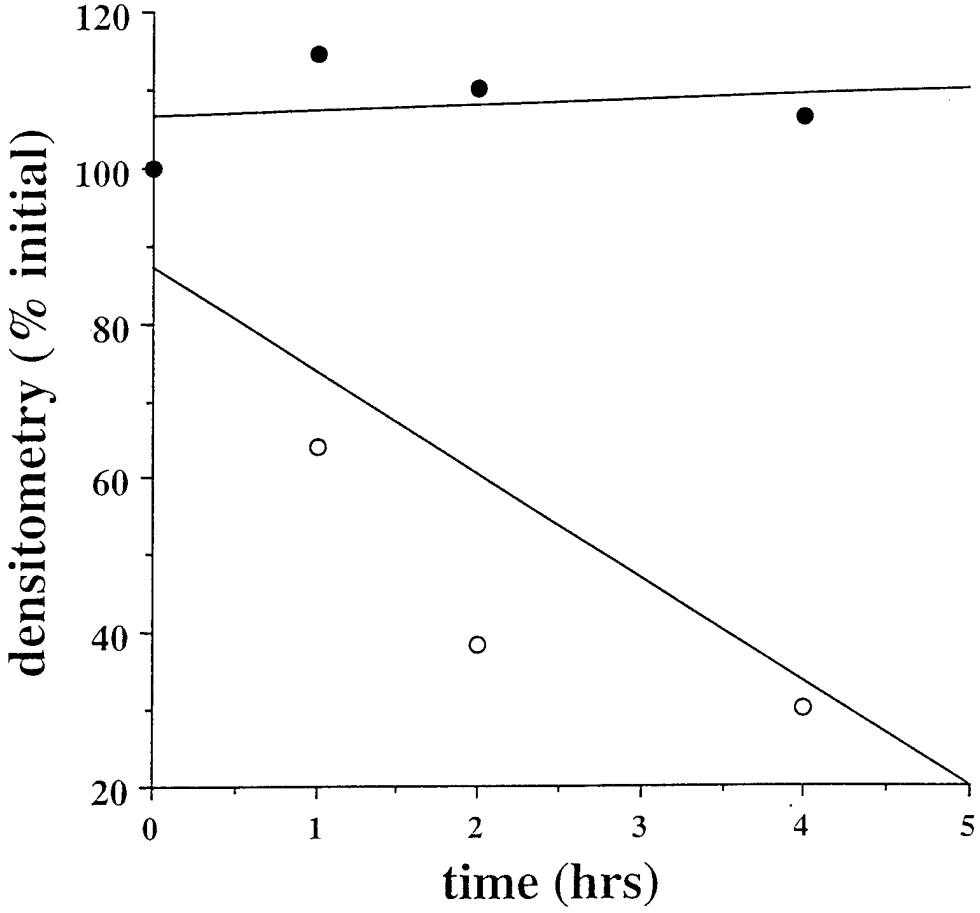
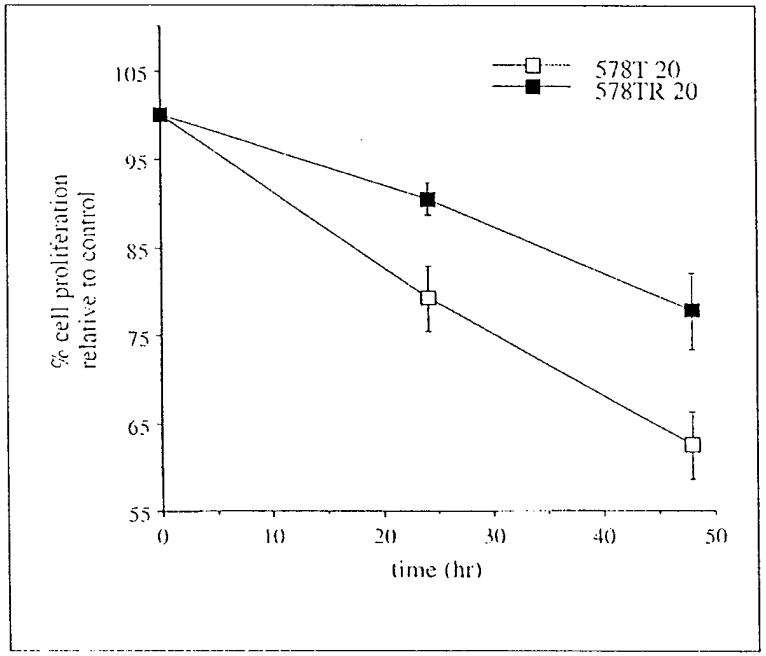


Figure 3B

A



Plated 20% Confluent

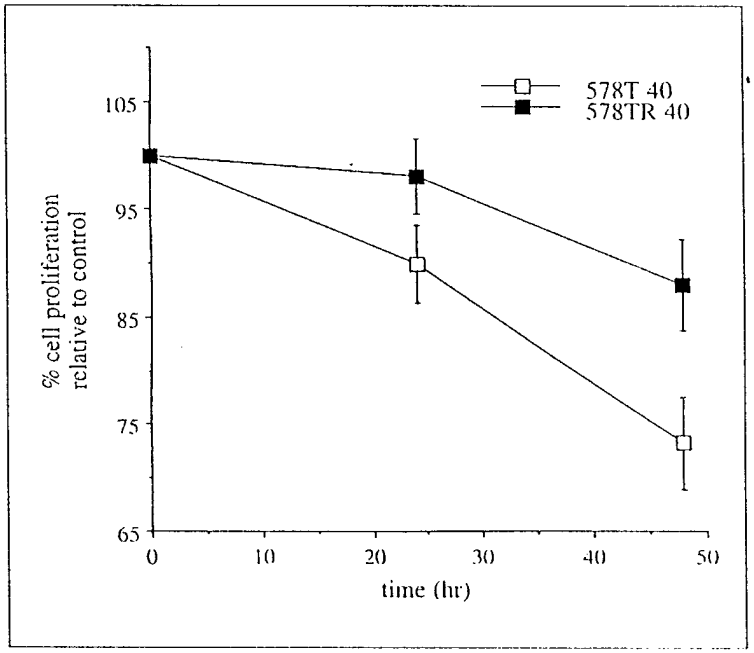


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Figure 4A

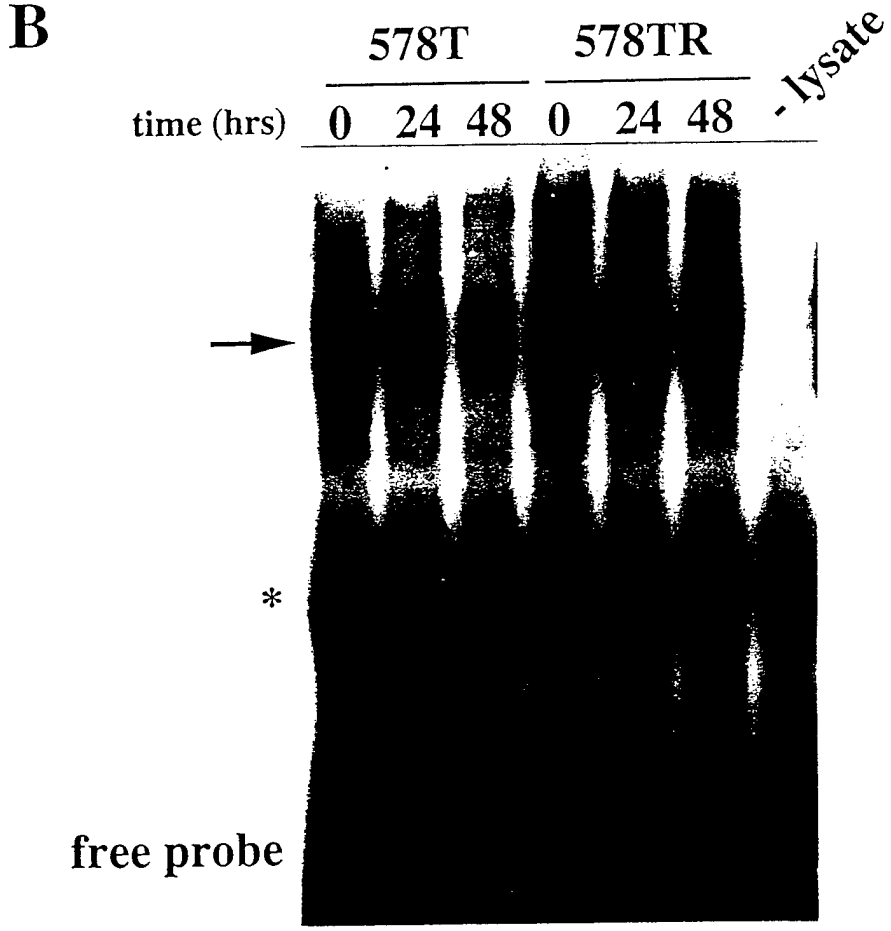

Figure 4B

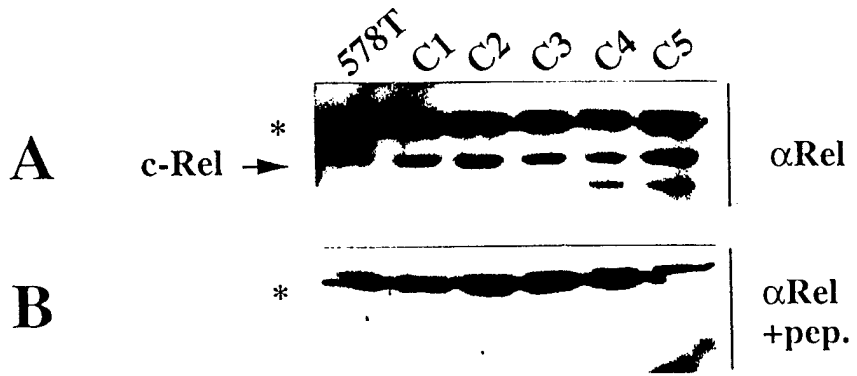

Figure 5 A&B

C

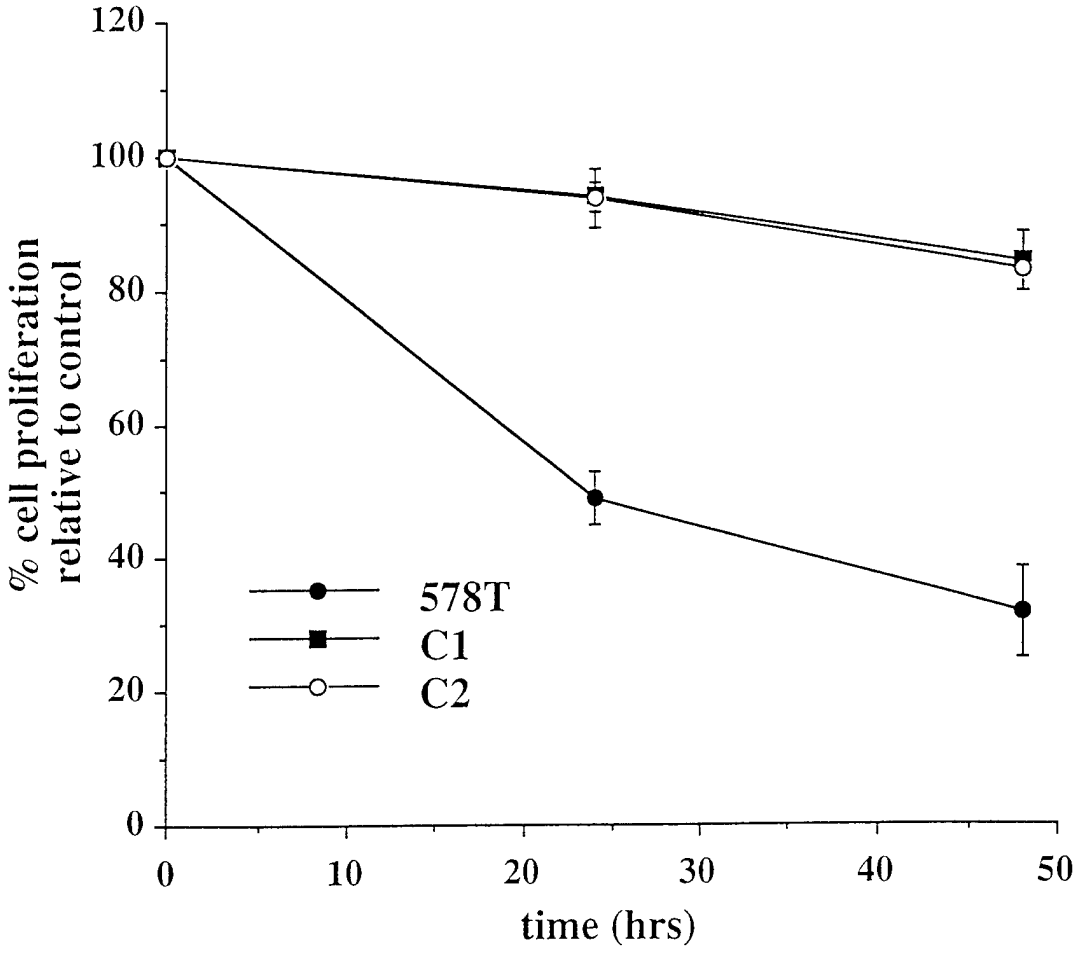
120
100
80
60
40
20
0
% cell proliferation relative to control
578T
C1
C2
0
10
20
30
40
50
time (hrs)

Figure 5C

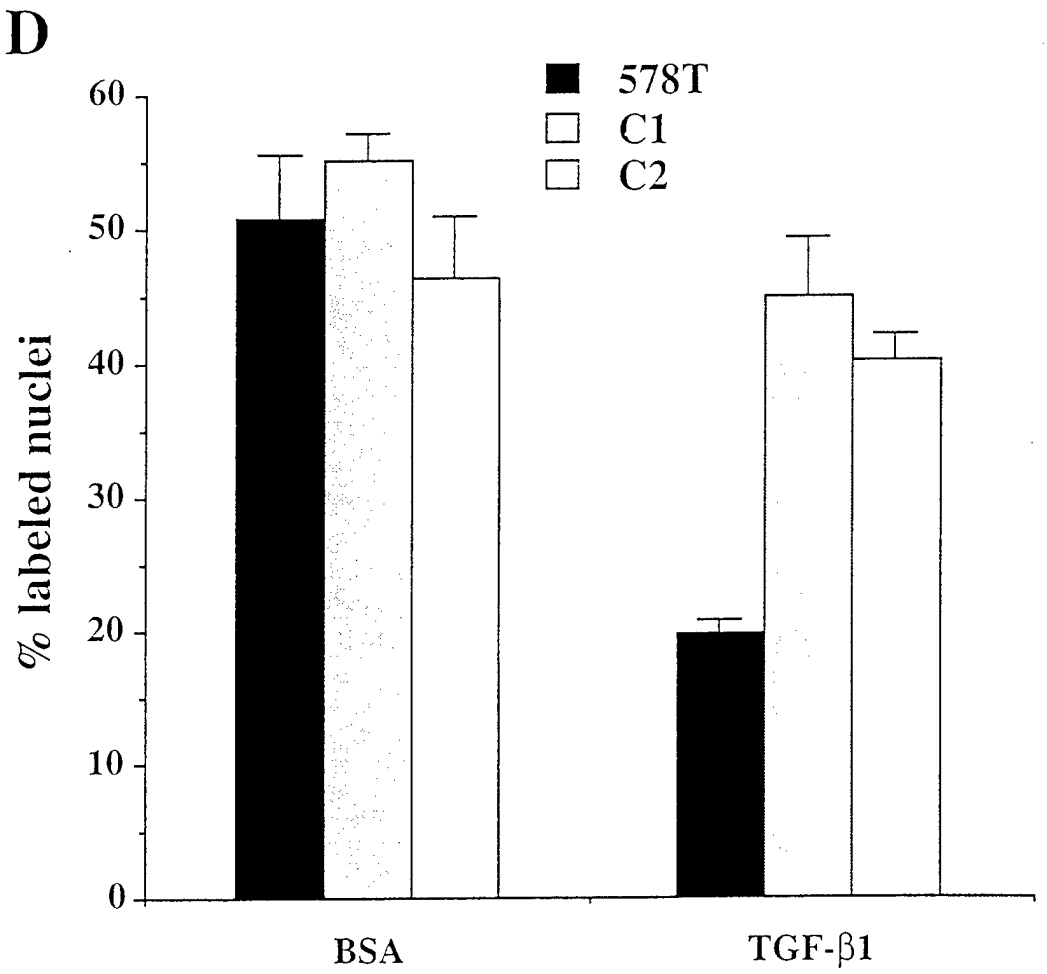

Figure 5D

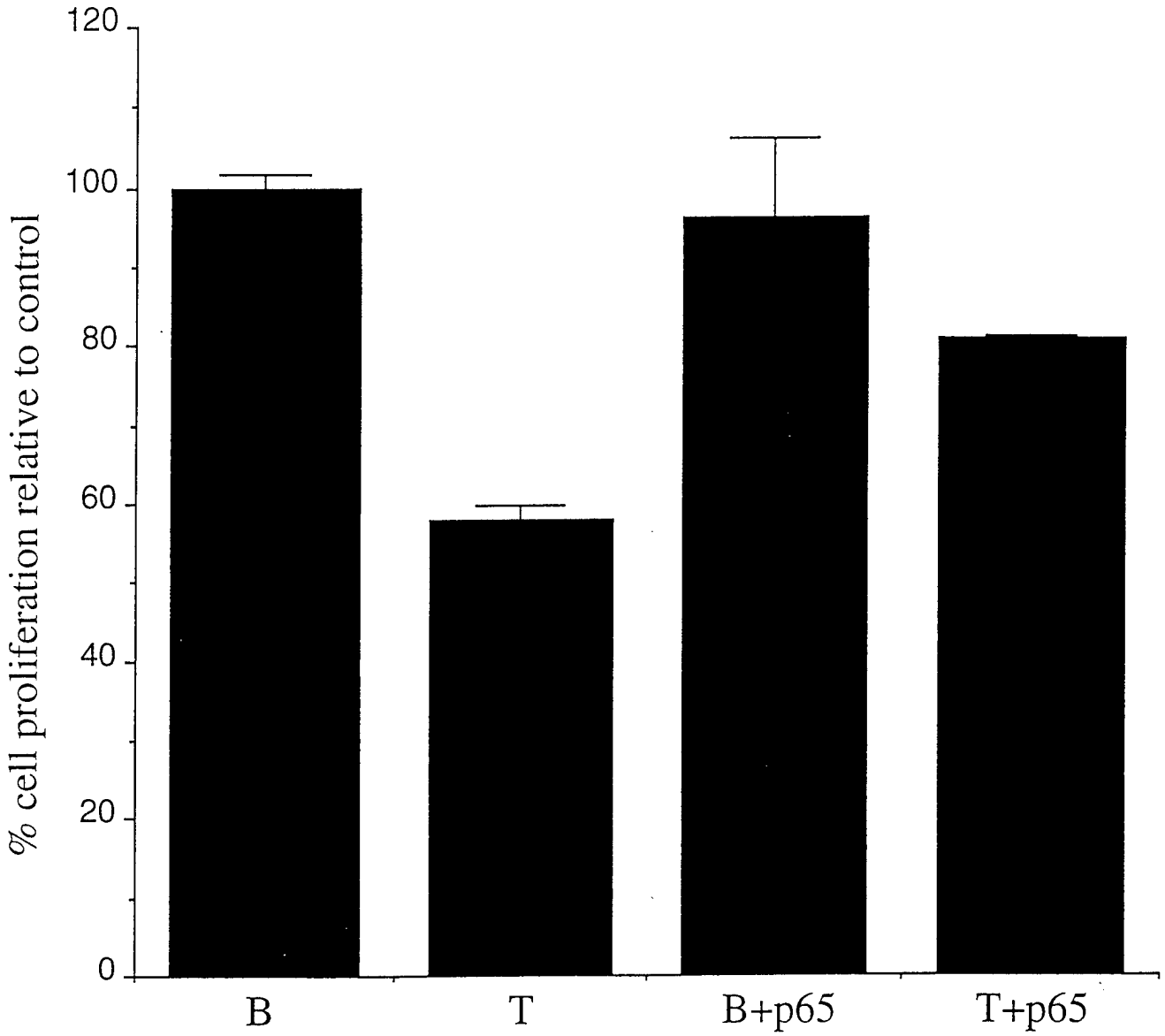
120
100
80
60
40
20
0
% cell proliferation relative to control
B
T
B+p65
T+p65

Figure 6